A PROGRAMMING ROLE FOR INTERLEUKIN-2 IN THE DIFFERENTIATION OF EFFECTOR AND MEMORY $CD8^+$ T CELLS, UNIQUE FROM IL-15 AND INDEPENDENT OF STAT5

by

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ABSTRACT

Environmental signals, including cytokines, received by $CD8^+$ T cells during the primary response to acute infection shape their commitment to effector and memory $CD8^+$ T cell fates, as well as their ability to respond to future pathogen challenges. The common gamma chain (γ_c) cytokine interleukin-2 (IL-2) provides a memory differentiation signal during the primary response that programs $CD8^+$ T cells for successful recall responses later. We find that although primary effector CTL development is modestly decreased in the absence of IL-2, the persistence of terminal effector phenotype and tissue residing memory $CD8^+$ T cell populations after pathogen clearance is greatly diminished. Furthermore, memory $CD8^+$ T cells generated in the absence of IL-2 signals are unable to undergo secondary effector CTL differentiation. We conclude that IL-2 promotes entry into and survival within an effector $CD8^+$ T cell differentiation program.

The role of IL-2 in promoting primary and secondary effector CTL differentiation is not shared by the highly related cytokine, IL-15. Although IL-15 supports the survival of effector phenotype $CD8^+$ T cells after pathogen clearance, its absence does not impair either primary or secondary effector CTL differentiation, nor does it impact the differentiation of long-term effector memory $CD8^+$ T cells. Thus, there is a unique role for IL-2, but not IL-15, in promoting the differentiation of effector CTL and programming memory $CD8^+$ T cells capable of secondary effector differentiation.

How $CD8^+$ T cells integrate IL-2 signals and the molecular nature of these signals in the development of functional memory populations is not understood. Because IL-2 induces potent activation of the STAT5 transcription factor, we tested the role of STAT5 in $CD8^+$ effector and memory differentiation. We find that STAT5 activity is broadly important for the expansion and effector functions of all effector CTL subsets during the primary response. Despite a broad role in expansion, the requirement for STAT5 was particularly important for survival of terminal effector phenotype and tissue residing memory $CD8^+$ T cells after pathogen clearance. Surprisingly, although STAT5 was important in primary effector CTL responses, and unlike IL-2, STAT5 activity is not required for the development of memory $CD8^+$ T cells capable of robust secondary expansion and secondary effector differentiation upon rechallenge. These findings highlight differential requirements for survival signals between primary and secondary effector CTL, and subsets of memory $CD8^+$ T cells. Furthermore, we demonstrate that IL-2 dependent programming of memory $CD8^+$ T cells capable of protective recall responses is STAT5 independent.

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CHAPTER 1

INTRODUCTION

CD8+ T cell response to acute infection

Naïve CD8+ T cells circulate through the host lymphoid system, continually sampling antigens being presented by antigen presenting cells in the secondary lymphoid organs. Upon encounter with cognate antigen, CD8+ T cells undergo a period of massive expansion, lasting approximately 7-8 days in the case of acute infection[1]. During this time, in which these CD8+ T cells are referred to as effector CD8+ T cells or cytolytic T cells (CTLs), they acquire essential effector functions required for pathogen clearance. These effector functions include the ability to migrate to the site of infection, specific killing of infected target cells by cytolysis, involving granzymes and perforin or via Fas/FasL, and the secretion of pro-inflammatory cytokines, such as IFN-γ and TNF-α[2]. After the pathogen is cleared, a period of contraction ensues, in which nearly all of the effector CD8+ T cell pool will die by apoptosis. However, a small percentage (approximately 5-10%) of the CD8+ T cells will survive and go on to form a long-lived population of memory CD8+ T cells[1].

Hallmarks of memory CD8+ T cells

A defining feature of adaptive immunity is immunological memory, which results in protection of the host from reinfection with the same or related pathogens. Memory CD8+ T cells that form after primary exposure to antigen have several properties that distinguish them from naïve CD8+ T cells that have no previous antigen exposure. First, memory CD8+ T cells are present in the host at higher frequencies than naïve CD8+ T cells. These memory populations are able to persist for long periods, often the lifetime of the host, through self-renewal via slow homeostatic turnover. Upon secondary exposure to cognate antigen, memory CD8+ T cells rapidly expand and reacquire their effector functions (recall). The combined result of these memory CD8+ T cell properties is the clearance of pathogen upon subsequent exposure, often before the host experiences

symptoms of infection. The factors regulating longevity of memory CD8+ T cells and optimal CD8+ T cell recall responses are of great interest, yet are not fully understood.

Models of memory CD8+ T cell differentiation and CD8+ T cell fate decisions

How a small fraction of memory CD8+ T cells is selected to survive after pathogen clearance while the majority of the effector CD8+ T cell pool dies is not understood. After initial antigen encounter, CTL differentiation and proliferation occur independent of antigen, and the development of a memory CD8+ T cell population [3]. It has been shown that a single CD8+ T cell is able to give rise to both effector and memory CD8+ T cell pools [4, 5], demonstrating that memory potential is not pre-programmed. Studies in which antigen presentation is curtailed show that commitment to memory CD8+ T cell lineage occurs early during an immune response, likely within the first 1-3 days of antigen exposure [6, 7]. Moreover, the composition of the effector and memory CD8+ T cell pools is heterogeneous in regards to cell surface phenotype, expression of effector molecules, long-term survival and turnover, and proliferative potential during recall [8, 9].

Several models have been proposed to describe the differentiation of protective memory CD8+ T cells from the effector CD8+ T cell pool [10]. The existence of multiple subsets of effector and memory CD8+ T cells with different longevity suggests that memory CD8+ T cell differentiation is an ongoing process during the effector phase into contraction, and into the early memory phase [11, 12]. Of the proposed models, the most likely scenario is a model in which CD8+ T cells commit to a terminal fate (death after pathogen clearance) or memory fate (potential to seed the memory CD8+ T cell pool) early during the immune response. Additional environmental signals likely promote a more or less differentiated effector state within these populations, and those CD8+ T cells

that have the potential to seed the memory pool will still undergo progressive differentiation over time after pathogen clearance during which time they will ultimately acquire the hallmarks of protective, fully differentiated memory $CD8^+$ T cell.

Consistent with such a model, it has been shown that at the peak of the effector $CD8^+$ T cell response to the acute virus LCMV-Armstrong, two distinct populations within the effector $CD8^+$ T cell pool can be distinguished that differ in their longevity within the memory $CD8^+$ T cell pool [13]. These two populations can be distinguished based on differential expression of the cell surface molecules KLRG1 and IL7Rα (CD127) [13-15]. $CD8^+$ T cells falling in one population, expressing high levels of KLRG1 and low levels of IL7Rα, herein called terminal effector phenotype $CD8^+$ T cells, gradually decline after pathogen clearance [13]. $CD8^+$ T cells falling in the second population, termed memory precursor or memory phenotype effector $CD8^+$ T cells, express low levels of KLRG1 and high levels of IL7Rα. This population is enriched for the potential to seed the memory $CD8^+$ T cell pool, has more robust proliferative capacity than terminal effectors and over time, the circulating memory $CD8^+$ T cell pool will evolve to be nearly entirely composted of this phenotype. The existence of such populations shows that signals received by effector $CD8^+$ T cells during the primary response after antigen stimulation regulate $CD8^+$ T cell effector and memory fate decisions.

While the differential expression of KLRG1 and IL7Rα are useful markers for identifying $CD8^+$ T cells that have increased long lived memory potential, they do not themselves drive $CD8^+$ T cell differentiation, but instead are indicative of fate decisions made earlier. Forcing expression of IL7Rα by effector $CD8^+$ T cells does not increase memory potential [16, 17]. KLRG1 was found to be upregulated on terminally differentiated $CD8^+$ T cells following LCMV infection of mice [18]. These $KLRG1^{hi}$ $CD8^+$ T cells were potent effector CTL, but were resistant to secondary proliferation upon re-exposure to antigen. It was subsequently shown to be upregulated within $CD8^+$ T cells

that had low IL7Rα expression [13]. Knockdown of KLRG1 expression in $CD8^+$ T cells had no effect on their effector differentiation[13]. In addition, not all of the $CD8^+$ T cells with an $IL7Ra^{hi}$ phenotype will survive to seed the memory $CD8^+$ T cell pool, and the $CD8^+$ T cells within this subset also change in their secondary proliferative potential as they mature into the memory phase [14, 15]. Moreover, it is unclear to what extent these different subpopulations are able to protect from secondary challenge. Although terminal effector phenotype $CD8^+$ T cells have less proliferative potential, they readily exert their effector functions [18] and thus may provide a frontline of protection from reinfection.

In addition to the heterogeneity seen within the effector $CD8^+$ T cell pool, different subsets of $IL7R\alpha^{hi}$ memory $CD8^+$ T cells emerge as well [1, 8-10]. In general, memory $CD8^+$ T cells can be divided into two subsets, central memory (T_{CM}) and effector memory (T_{EM}) which differ in their anatomical location, cell surface phenotype, expression of effector molecules, and proliferative potential. Broadly, T_{CM} are found within the secondary lymphoid organs, are $CD62L^{hi}CCR7^{hi}$, do not immediately express effector molecules, and have high proliferative potential. T_{EM}, on the other hand, are found in peripheral nonlymphoid tissues, are $CD62L^{lo}CCR7^{lo}$, display some effector molecule expression, and have low proliferative potential. However, memory $CD8^+$ T cells with mixed phenotypes also exist, and expression of these two markers may not be the best predictor of proliferative and/or protective potential upon recall [19]. Moreover, the lineage relationship of effector and memory $CD8^+$ T cell subsets as well as the actual protective capacity of T_{CM} versus T_{EM}, remain controversial and unclear.

Much progress has been made in identifying subsets present within effector and memory $CD8^+$ T cell pools. However, the signals that drive formation of these subsets and the signals that regulate $CD8^+$ T cell fate decisions are still not well understood. In addition, the degree of plasticity within commitment to a particular effector or memory

$CD8^+$ T cell subset, the permanence or irreversibility of lineage commitment, and the signals that may be involved in commitment, are not well understood. It is possible that after initial commitment, inflammation or cytokines in the environment could serve to further drive $CD8^+$ T cells toward a more differentiated state [13]. It is also worth noting that although common model systems are used to study in vivo T cell responses and memory formation, it is still unclear if for different infectious conditions, if common and/or distinct pathways are used in $CD8^+$ T cell fate decisions.

Many signals converge on $CD8^+$ T cells during an immune response. These include signals through the T cell receptor (TCR) delivered by antigen stimulation, costimulatory signals from antigen-presenting cells, and signals from cytokines and Type I interferons via receptors expressed on $CD8^+$ T cells. While TCR signal and costimulation are essential for initiation of $CD8^+$ T cell responses, as discussed above, a relatively brief TCR stimulus is adequate to result in effector and memory $CD8^+$ T cell generation [6, 7]. In addition, the $CD8^+$ T cell TCR repertoire is similarly represented during effector and memory phases [20], suggesting that differences in TCR affinity do not regulate $CD8^+$ T cell memory development. Thus, signals derived from the environment during the primary response to infection, including inflammatory signals and those transmitted via cytokines and cytokine receptors, play a significant role in regulating $CD8^+$ T cell fate decisions.

Transcription Factors in $CD8^+$ T cell fate decisions

Several transcription factors have been shown to have crucial roles in $CD8^+$ T cell effector differentiation and effector versus memory fate decisions. These transcription factors are associated with either terminally differentiated effector or less differentiated long-lived memory $CD8^+$ T cell states. In the case of T-bet [13, 21-24] and Blimp-1 [25-28], increased expression of these transcription factors promotes a more differentiated

terminal effector state in CD8+ T cells. On the other hand, EOMES [22, 24, 29, 30] and Bcl-6 [25, 31, 32] are associated with establishment of memory CD8+ T cell populations and opposition of terminal effector differentiation.

It has been shown in the case of T-bet that the pro-inflammatory cytokine IL-12 can increase expression levels of T-bet and promote a more differentiated effector state in CD8+ T cells [13]. However, a direct connection between environmental signals, such as cytokines, received by CD8+ T cells during an immune response and the resulting transcriptional outcome driving differentiation towards short-lived effector versus memory precursor fate is not clear. Although it is well established that several of these transcription factors are known to bind and regulate the promoters of genes regulating CTL function, such as Perforin, GzB, and IFNγ, how the transcriptional targets of these factors plays into resulting fate decisions in unknown. It is not clear if these transcription factors promote effector vs memory differentiation by regulating genes that actually drive differentiation, or if they promote memory CD8+ T cell survival and secondary effector differentiation by allowing or restricting access to essential transcriptional programs.

IL-2 signaling in programming of CD8+ T cell memory

Common gamma chain (γ_c) cytokine family members are defined by their common use of the type I cytokine receptor subunit, γ_c (CD132), expressed on several leukocytes, including T cells. This family includes several cytokines, which have been shown to play a role in naïve CD8+ T cell homeostasis and during CD8+ T cell effector and memory phases of the immune response [33]. Regulation of the specific subunits for each cytokine varies, resulting in different cytokine-specific stimulation of CD8+ T cells through the course of an immune response.

The cytokine IL-2, a member of the γ_c cytokine family, is of interest in $CD8^+$ T cell fate decisions and the main topic of this thesis. IL-2 was originally named T cell growth factor because it resulted in proliferation of activated $CD8^+$ T cells in vitro [34, 35]. Surprisingly, initial studies regarding the in vivo role for IL-2 in promoting T cell proliferation and effector differentiation suggested that IL-2 was not required for successful primary T cell responses to infection [36-38]. However, these in vivo studies of IL-2 in $CD8^+$ T cell responses to infection were complicated and difficult to interpret due to the requirement of IL-2 for maintenance of regulatory T cells [39], and the resulting development of lympho-proliferation and autoimmunity the absence of IL-2 or components of the IL-2 receptor [40-43]. The development of model systems where IL-2 receptor components are absent, but a normal immune environment is present, has allowed revisiting of the role of IL-2 in effector and memory $CD8^+$ T cell fate decisions [44, 45].

$CD4^+$ T cell help has been shown to be required for memory $CD8^+$ T cell programming [46-49]. In an attempt to understand how $CD4^+$ T cells provide help to $CD8^+$ T cells, the cytokine IL-2 was investigated as a potential driver of memory $CD8^+$ T cell programming [45]. IL-2 is expressed at high levels mainly by activated $CD4^+$ T cells, and to a lesser degree by $CD8^+$ T cells and dendritic cells, during an active immune response. In vivo examination of IL-2 production have shown that the production of IL-2 peaks at 5-6 hours postantigen challenge, and is no longer detected after 16 hours [50, 51]. In addition, expression of the trimeric IL-2R complex on activated $CD8^+$ T cells (described in more detail below) is transient. The result is a short window of time in which $CD8^+$ T cells actively receive strong IL-2 signals early on during an immune response.

Using an in vivo model with a normal immune environment, it was found that $CD8^+$ T cells that do not receive IL-2 signals during primary immune response show only

modestly reduced levels of proliferation and effector $CD8^+$ T cell expansion and readily formed long-lived memory $CD8^+$ T cell populations upon pathogen clearance [44, 45]. However, memory $CD8^+$ T cells generated in the absence of IL-2 were phenotypically different than wildtype $CD8^+$ T cells in that they skewed towards a central memory phenotype [45]. Most strikingly, memory $CD8^+$ T cells generated in the absence of IL-2 signals had defective recall responses upon secondary challenge [45]. This points to an essential role for the cytokine IL-2 in programming of memory $CD8^+$ T cells capable of protective recall responses. Moreover, this suggests that the recall capacity of memory $CD8^+$ T cells (programming) is induced distinctly from survival of $CD8^+$ T cells into the memory phase.

IL-2 receptor signaling

The IL-2 receptor (IL-2R) is a trimeric complex consisting of the three subunits IL-2Rα(CD25), IL-2Rβ(CD122), and the common gamma chain, γ_c (CD132). Expression of the three subunits is regulated differently, and the trimeric receptor is not present on resting $CD8^+$ T cells. Instead, the basal expression of β and γ_c subunits needs to be supplemented with expression of the high affinity receptor subunit, IL-2Rα[39], which occurs transiently at high levels upon $CD8^+$ T cell activation[52]. After its expression is induced, the high affinity IL-2Rα subunit of the trimeric IL-2 receptor complex binds to IL-2 and subsequently associates with β and γ_c subunits, forming a stable quaternary complex [53, 54]. Although binding of IL-2 to the IL-2Rβ and γ_c subunits has been observed in vitro [55], it appears that this is unlikely to have an in vivo biological role, as concentrations of IL-2 required for functional signaling through the dimeric receptor are much higher than is found in vivo [56]. Moreover, IL-2-/- and CD25-/- mice develop similar autoimmune phenotypes [42, 43]. Thus, expression of IL-2Rα confers the ability of $CD8^+$ T cells to receive IL-2 signals. After the resolution of inflammation, $CD8^+$ T cells

downregulate expression of CD25 and IL-2 is no longer robustly produced. The result is strong yet transient signals received by $CD8^+$ T cells during the effector phase of an immune response.

Signal transduction through the IL-2R occurs via the IL-2Rβ and γ_c receptor subunits [56, 57]. The Janus Kinase (JAK) family members JAK1 and JAK3 constitutively associate with the cytoplasmic tails of IL-2Rβ and γ_c, respectively. JAK1 and JAK3 are activated immediately by autophosphorylation following receptor activation, and they subsequently trans-phosphorylate each other and also phosphorylate several tyrosine residues on the cytoplasmic tails of IL-2Rβ and γ_c. This creates docking sites for signal transducer and activator of transcription family member 5a and 5b, two mainly redundant isoforms of STAT5 in $CD8^+$ T cells [58]. The associated STAT5 is then phosphorylated, mainly by JAK3, resulting in dimerization and translocation to the nucleus where it acts as a transcription factor and regulates gene expression important in T cell proliferation, survival, and effector differentiation. STAT3 is also recruited to phosphorylated tyrosines on the IL-2Rβ cytoplasmic tail, where it is phosphorylated and activated, but to a lesser extent than STAT5 [59, 60].

In addition to association with STAT5 and STAT3, the phosphorylated tail of IL-2Rβ also associates with Shc, resulting in activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [56, 57]. Some reports have suggested that PI3K may be recruited to specific phosphorylated tyrosines on IL-2Rβ cytoplasmic tail directly, and another report suggests activation of the PI3K pathway independent of cytoplasmic tyrosines on IL-2Rβ (reviewed in [56]). Activation of the MAPK and PI3K pathways by IL-2 have been shown to be important in promoting T cell survival and proliferation [39, 56].

Topics addressed in this dissertation

In Chapter 2, a thorough examination of effector CD8$^+$ T cell responses in the absence of IL-2 is performed. The result is an enhanced understanding of the role of IL-2 in effector and memory CD8$^+$ T cell fate decisions. As previously shown, the expansion of CD8$^+$ T cells in the absence of IL-2 signals was only modestly reduced. However, we found that IL-2 selectively drives differentiation and survival of terminal effector phenotype CTL during the primary response to acute infection, and the entire difference in numbers during the effector phase could be attributed to the reduced formation of terminal CD8$^+$ T cells in the absence of IL-2. Memory precursor and long-lived memory CD8$^+$ T cells were established normally in peripheral lymphoid organs in the absence of IL-2 signals. Closer examination of memory CD8$^+$ T cells in the spleen and peripheral tissues revealed a role for IL-2 in the differentiation of effector memory CD8$^+$ T cells.

We also further analyzed the defective recall response of CD8$^+$ T cells generated in the absence of IL-2 signals. We found that in addition to failure of accumulation of secondary effector CD8$^+$ T cells upon rechallenge, differentiation of secondary effector CTL to terminal effector phenotype was more severe than seen in the primary response. We conclude that IL-2 signals during the primary response allow entry into and survival within a CD8$^+$ T cell effector differentiation program.

Although reduced terminal effector phenotype CTL differentiation during the primary response is seen in the absence of IL-2 signals, this population is not completely absent. Potential compensation for the lack of IL-2 signals by the closely related γ_c family member IL-15 is addressed in Chapter 3. IL-2 and IL-15 are closely related cytokines, with receptors sharing two of three subunits, IL-2Rβ (CD122) and γ_c (CD132), which results in induction of signaling pathways shared by both cytokines [61].

Prior studies have suggested that IL-2 and IL-15 differentially regulate certain aspects of CD8$^+$ memory T cell differentiation. While activation in the presence of high

levels of IL-2 in vitro preferentially promotes the subsequent in vivo development of effector and effector memory T cells, activation in the presence of IL-15 preferentially promotes central memory differentiation [62, 63]. Both of these cytokines have been used or proposed as potential immunotherapeutics. High-dose IL-2 treatment has been used clinically to treat several types of cancer, including renal cell carcinoma and melanoma, with modest effects on a subset of recipients [64-66]. The use of IL-15 has been suggested for boosting T and NK cell anti-tumor responses and as a vaccination adjuvant in various model systems [61, 67-72]. While IL-15 has a well-described role in promoting the homeostasis and survival of $CD8^+$ memory T cells [73], differing mouse models of acute infection demonstrate either no role [74, 75] or a significant role[76] for IL-15 in the generation of effector CTL responses. In all, it remains unclear how and to what extent IL-2 and IL-15 mediate overlapping, differing or even opposing functions, particularly in the early phases of activation in which T cells enter into their differentiation program [3, 77].

We found that IL-15 does not drive the differentiation of terminal effector phenotype $CD8^+$ T cells during the primary effector response in our model system. In addition, we found that IL-15 does not have a role in memory $CD8^+$ T cell programming. Instead, we conclude that IL-15 is a survival signal for effector and memory $CD8^+$ T cells after pathogen clearance. These results indicate that even with a shared cytokine signaling apparatus, IL-2 signals in effector and memory $CD8^+$ T cell programming are unique from those of IL-15.

Chapter 4 addresses the molecular nature of the IL-2 signal as received by $CD8^+$ T cells. The mechanisms by which $CD8^+$ T cells integrate environmental signals resulting in effector and memory fate decisions are not understood. In this chapter, the JAK3/STAT5 pathway is tested for its role in driving $CD8^+$ T cell fate decisions and programming of memory $CD8^+$ T cells.

IL-2 induces several kinase pathways (JAK/STAT, PI3K, MAPK, as described above). All of these pathways are capable of induction by signals received through other receptors during an immune response, at least to some degree. However, the JAK3/STAT5 pathway is induced by γ_c cytokines alone, and of these, IL-2 is the most potent stimulant for JAK3/STAT5 activation during the effector $CD8^+$ T cell response. For this reason, we chose to study the role of STAT5 in effector and memory $CD8^+$ T cell differentiation, and in memory $CD8^+$ T cell programming. We found that unlike IL-2, STAT5 does not drive selective differentiation of a particular effector $CD8^+$ T cell subset. Instead, STAT5 signals are broadly important for full accumulation of all effector $CD8^+$ T cells and their effector function during the primary immune response. After pathogen clearance, STAT5 signals are particularly important for the survival of terminal effector phenotype CTL and the establishment and maintenance of tissue residing memory $CD8^+$ T cells. Importantly, we found that IL-2 driven programming of memory $CD8^+$ T cells is independent of STAT5, as STAT5 was not required for robust recall responses by memory $CD8^+$ T cells. This highlights different requirements for STAT5 mediated survival signals for primary and secondary effector $CD8^+$ T cell responses and demonstrate that IL-2 dependent programming of memory $CD8^+$ T cells is STAT5 independent.

References

1. Williams, M.A. and M.J. Bevan, *Effector and memory CTL differentiation.* Annual review of immunology, 2007. **25**: p. 171-92.

2. Lewinsohn, D.A., M.C. Gold, and D.M. Lewinsohn, *Views of immunology: effector T cells.* Immunological reviews, 2011. **240**(1): p. 25-39.

3. Kaech, S.M. and R. Ahmed, *Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells.* Nature immunology, 2001. **2**(5): p. 415-22.

4. Stemberger, C., et al., *A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets.* Immunity, 2007. **27**(6): p. 985-97.

5. Schepers, K., et al., *Dissecting T cell lineage relationships by cellular barcoding.* The Journal of experimental medicine, 2008. **205**(10): p. 2309-18.

6. Mercado, R., et al., *Early programming of T cell populations responding to bacterial infection.* Journal of immunology, 2000. **165**(12): p. 6833-9.

7. Prlic, M., G. Hernandez-Hoyos, and M.J. Bevan, *Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response.* The Journal of experimental medicine, 2006. **203**(9): p. 2135-43.

8. Jameson, S.C. and D. Masopust, *Diversity in T cell memory: an embarrassment of riches.* Immunity, 2009. **31**(6): p. 859-71.

9. Lefrancois, L. and A.L. Marzo, *The descent of memory T-cell subsets.* Nature reviews. Immunology, 2006. **6**(8): p. 618-23.

10. Kaech, S.M. and E.J. Wherry, *Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection.* Immunity, 2007. **27**(3): p. 393-405.

11. Williams, M.A. and M.J. Bevan, *Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells.* Journal of immunology, 2004. **173**(11): p. 6694-702.

12. Masopust, D., et al., *The role of programming in memory T-cell development.* Current opinion in immunology, 2004. **16**(2): p. 217-25.

13. Joshi, N.S., et al., *Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor.* Immunity, 2007. **27**(2): p. 281-95.

14. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells.* Nature immunology, 2003. **4**(12): p. 1191-8.

15. Huster, K.M., et al., *Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(15): p. 5610-5.

16. Haring, J.S., et al., *Constitutive expression of IL-7 receptor alpha does not support increased expansion or prevent contraction of antigen-specific CD4 or CD8 T cells following Listeria monocytogenes infection.* Journal of immunology, 2008. **180**(5): p. 2855-62.

17. Hand, T.W., M. Morre, and S.M. Kaech, *Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(28): p. 11730-5.

18. Voehringer, D., et al., *Viral infections induce abundant numbers of senescent CD8 T cells.* Journal of immunology, 2001. **167**(9): p. 4838-43.

19. Hikono, H., et al., *Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells.* The Journal of experimental medicine, 2007. **204**(7): p. 1625-36.

20. Slifka, M.K. and J.L. Whitton, *Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR.* Nature immunology, 2001. **2**(8): p. 711-7.

21. Yeo, C.J. and D.T. Fearon, *T-bet-mediated differentiation of the activated CD8+ T cell.* European journal of immunology, 2011. **41**(1): p. 60-6.

22. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin.* Nature immunology, 2005. **6**(12): p. 1236-44.

23. Sullivan, B.M., et al., *Antigen-driven effector CD8 T cell function regulated by T-bet.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(26): p. 15818-23.

24. Cruz-Guilloty, F., et al., *Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs.* The Journal of experimental medicine, 2009. **206**(1): p. 51-9.

25. Crotty, S., R.J. Johnston, and S.P. Schoenberger, *Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation.* Nature immunology, 2010. **11**(2): p. 114-20.

26. Kallies, A., et al., *Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses.* Immunity, 2009. **31**(2): p. 283-95.

27. Rutishauser, R.L., et al., *Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties.* Immunity, 2009. **31**(2): p. 296-308.

28. Shin, H., et al., *A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection.* Immunity, 2009. **31**(2): p. 309-20.

29. Banerjee, A., et al., *Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche.* Journal of immunology, 2010. **185**(9): p. 4988-92.

30. Pearce, E.L., et al., *Control of effector CD8+ T cell function by the transcription factor Eomesodermin.* Science, 2003. **302**(5647): p. 1041-3.

31. Ichii, H., et al., *Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells.* Nature immunology, 2002. **3**(6): p. 558-63.

32. Ichii, H., et al., *Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells.* Journal of immunology, 2004. **173**(2): p. 883-91.

33. Rochman, Y., R. Spolski, and W.J. Leonard, *New insights into the regulation of T cells by gamma(c) family cytokines.* Nature reviews. Immunology, 2009. **9**(7): p. 480-90.

34. Morgan, D.A., F.W. Ruscetti, and R. Gallo, *Selective in vitro growth of T lymphocytes from normal human bone marrows.* Science, 1976. **193**(4257): p. 1007-8.

35. Smith, K.A., *Interleukin-2: inception, impact, and implications.* Science, 1988. **240**(4856): p. 1169-76.

36. Wei, Y., et al., *Dendritoma vaccination combined with low dose interleukin-2 in metastatic melanoma patients induced immunological and clinical responses.* International journal of oncology, 2006. **28**(3): p. 585-93.

37. Kundig, T.M., et al., *Immune responses in interleukin-2-deficient mice.* Science, 1993. **262**(5136): p. 1059-61.

38. Bachmann, M.F., et al., *Antiviral immune responses in mice deficient for both interleukin-2 and interleukin-4.* Journal of virology, 1995. **69**(8): p. 4842-6.

39. Malek, T.R., *The biology of interleukin-2.* Annual review of immunology, 2008. **26**: p. 453-79.

40. Sadlack, B., et al., *Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene.* Cell, 1993. **75**(2): p. 253-61.

41. Sadlack, B., et al., *Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells.* European journal of immunology, 1995. **25**(11): p. 3053-9.

42. Willerford, D.M., et al., *Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment.* Immunity, 1995. **3**(4): p. 521-30.

43. Suzuki, H., et al., *Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta.* Science, 1995. **268**(5216): p. 1472-6.

44. Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells.* Journal of immunology, 2010. **184**(12): p. 6719-30.

45. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells.* Nature, 2006. **441**(7095): p. 890-3.

46. Janssen, E.M., et al., *CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes.* Nature, 2003. **421**(6925): p. 852-6.

47. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.

48. Sun, J.C. and M.J. Bevan, *Defective CD8 T cell memory following acute infection without CD4 T cell help.* Science, 2003. **300**(5617): p. 339-42.

49. Sun, J.C., M.A. Williams, and M.J. Bevan, *CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection.* Nature immunology, 2004. **5**(9): p. 927-33.

50. Sojka, D.K., et al., *IL-2 secretion by CD4+ T cells in vivo is rapid, transient, and influenced by TCR-specific competition.* Journal of immunology, 2004. **172**(10): p. 6136-43.

51. D'Souza, W.N. and L. Lefrancois, *Frontline: An in-depth evaluation of the production of IL-2 by antigen-specific CD8 T cells in vivo.* European journal of immunology, 2004. **34**(11): p. 2977-85.

52. Rogers, W.O., et al., *Visualization of antigen-specific T cell activation and cytokine expression in vivo.* Journal of immunology, 1997. **158**(2): p. 649-57.

53. Rickert, M., et al., *The structure of interleukin-2 complexed with its alpha receptor.* Science, 2005. **308**(5727): p. 1477-80.

54. Stauber, D.J., et al., *Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(8): p. 2788-93.

55. Lin, J.X. and W.J. Leonard, *Signaling from the IL-2 receptor to the nucleus.* Cytokine & growth factor reviews, 1997. **8**(4): p. 313-32.

56. Gaffen, S.L., *Signaling domains of the interleukin 2 receptor.* Cytokine, 2001. **14**(2): p. 63-77.

57. Nelson, B.H. and D.M. Willerford, *Biology of the interleukin-2 receptor.* Advances in immunology, 1998. **70**: p. 1-81.

58. Hennighausen, L. and G.W. Robinson, *Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B.* Genes & development, 2008. **22**(6): p. 711-21.

59. Kovanen, P.E. and W.J. Leonard, *Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways.* Immunological reviews, 2004. **202**: p. 67-83.

60. Johnston, J.A., et al., *Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(19): p. 8705-9.

61. Waldmann, T.A., *The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design.* Nature reviews. Immunology, 2006. **6**(8): p. 595-601.

62. Manjunath, N., et al., *Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes.* The Journal of clinical investigation, 2001. **108**(6): p. 871-8.

63. Weninger, W., et al., *Migratory properties of naive, effector, and memory CD8(+) T cells.* The Journal of experimental medicine, 2001. **194**(7): p. 953-66.

64. Atkins, M.B., et al., *High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 1999. **17**(7): p. 2105-16.

65. McDermott, D.F., *Immunotherapy of metastatic renal cell carcinoma.* Cancer, 2009. **115**(10 Suppl): p. 2298-305.

66. Rosenberg, S.A., et al., *Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2.* JAMA : the journal of the American Medical Association, 1994. **271**(12): p. 907-13.

67. Halwani, R., et al., *Therapeutic vaccination with simian immunodeficiency virus (SIV)-DNA + IL-12 or IL-15 induces distinct CD8 memory subsets in SIV-infected macaques.* Journal of immunology, 2008. **180**(12): p. 7969-79.

68. Oh, S., et al., *Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(6): p. 3392-7.

69. Perera, L.P., et al., *Development of smallpox vaccine candidates with integrated interleukin-15 that demonstrate superior immunogenicity, efficacy, and safety in mice.* Journal of virology, 2007. **81**(16): p. 8774-83.

70. Leone, A., L.J. Picker, and D.L. Sodora, *IL-2, IL-7 and IL-15 as immuno-modulators during SIV/HIV vaccination and treatment.* Current HIV research, 2009. **7**(1): p. 83-90.

71. Yajima, T., et al., *Overexpression of interleukin-15 in vivo enhances antitumor activity against MHC class I-negative and -positive malignant melanoma through augmented NK activity and cytotoxic T-cell response.* International journal of cancer. Journal international du cancer, 2002. **99**(4): p. 573-8.

72. Kobayashi, H., et al., *Role of trans-cellular IL-15 presentation in the activation of NK cell-mediated killing, which leads to enhanced tumor immunosurveillance.* Blood, 2005. **105**(2): p. 721-7.

73. Surh, C.D. and J. Sprent, *Homeostasis of naive and memory T cells.* Immunity, 2008. **29**(6): p. 848-62.

74. Becker, T.C., et al., *Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells.* The Journal of experimental medicine, 2002. **195**(12): p. 1541-8.

75. Wherry, E.J., et al., *Homeostatic proliferation but not the generation of virus specific memory CD8 T cells is impaired in the absence of IL-15 or IL-15Ralpha.* Advances in experimental medicine and biology, 2002. **512**: p. 165-75.

76. Schluns, K.S., et al., *Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells.* Journal of immunology, 2002. **168**(10): p. 4827-31.

77. van Stipdonk, M.J., et al., *Dynamic programming of CD8+ T lymphocyte responses.* Nature immunology, 2003. **4**(4): p. 361-5.

CHAPTER 2

THE CYTOKINE IL-2 DRIVES ENTRY INTO AND SURVIVAL WITHIN AN EFFECTOR CD8+ T CELL DIFFERENTIATION PROGRAM *

 Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells.* Journal of Immunology, 2010. 184(12): p.6719-30.

Introduction

Antigen-specific $CD8^+$ T cells receive instructional signals during the primary phase of the immune response to acute infection that dictate later stages of differentiation. Upon antigen recognition, $CD8^+$ T cells undergo massive clonal expansion and acquire effector functions that are critical for the elimination of intracellular pathogens, including cytolytic function and the ability to produce proinflammatory cytokines such as IFNγ and TNFα. After the infection is resolved, most of the effector population dies, leaving behind a long- lived population of memory cells capable of rapid secondary protection upon re-exposure to the same or a related pathogen [1, 2].

$CD8^+$ memory T cell precursors can be identified among the effector population at the peak of the response to acute infection based on the expression of cell surface molecules such as IL-7Rα and KLRG1 [3, 4]. Intensive efforts are underway to understand the nature of the differentiation signals that differentially promote the emergence of effector cells that express high levels of KLRG1 and low levels of IL-7Rα (terminal effector phenotype $CD8^+$ T cells), and memory precursor cells that express low levels of KLRG1 and high levels of IL-7Rα. $CD4^+$ T cell-derived "help" is of particular importance in the generation of functional (capable of secondary responses to antigen) $CD8^+$ memory T cells [5-9]. Other studies have suggested that memory potential may depend at least in part on asymmetric division at the initiation of the T cell response [10, 11] or differential expression of the transcription factor T-bet driven by exposure to inflammatory cytokines such as IL-12 [3].

Our recent studies have focused on the role of IL-2 in $CD8^+$ memory T cell differentiation. Like others [12-14], we found that in the absence of IL-2 signals, $CD8^+$ T cells showed only modest impairment in their ability to make robust primary responses following acute infection. However, IL-2 signals during the primary response were

required for the ability of the ensuing $CD8^+$ memory cells to generate optimal secondary responses [15]. Several other observations indicated that the impact of IL-2 on $CD8^+$ T cells impacted multiple differentiation pathways. For example, memory T cells generated in the absence of IL-2 skewed to a central memory-like phenotype as measured by expression of CD62L and the ability to produce IL-2 upon restimulation [15].

We find that IL-2 plays a central role in the differentiation and survival of effector CTL that persist during the first few months after infection, as well as tissue- residing effector memory CTL. IL-2Rα-deficient $CD8^+$ effector T cells responding to acute infection display robust cytokine production but modest decreases in CTL activity. Upon secondary challenge, IL-2Rα-deficient $CD8^+$ memory T cells display a severe defect in their ability to differentiate into secondary effector CTL, maintaining an IL-$7R\alpha^{hi}$ $CD62L^{hi}$ phenotype and a cytokine production profile typical of memory CTL, not effector CTL.

Materials and methods

Mice and infections

6 to 8 week old C57BL/6 (B6), B6.129S4-Il2ratm1Dw (IL-2Rα-deficient), B6.SJL-PtprcaPepcb/BoyJ (B6.SJL, $Ly5.1^+$) and B6.PL-Thy1a/CyJ (B6.PL, $Thy1.1^+$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). WT and IL-2Rα- deficient P14 TCR transgenic mouse colonies were maintained at the University of Utah. All animal experiments were conducted with the approval of the IACUC committee at the University of Utah. LCMV Armstrong 53b was grown in BHK cells and titered in Vero cells [16]. Mice were infected intraperitoneally (i.p.) with 2×10^5 plaque-forming units (PFU). Recombinant *Listeria monocyt*ogenes expressing the LCMV GP33-41 peptide (Lm-gp33, generated using described methods) was propagated in BHI broth and agar plates as previously described [17-19]. Prior to infection, the bacteria were grown to log phase and

concentration determined by measuring the O.D. at 600 nm (O.D. of 1 = 1 x 10^9 CFU/ml). For secondary challenges, mice were injected intravenously (i.v.) with 2 x 10^5 colony forming units (CFU). Recombinant vaccinia virus expressing the LCMV glycoprotein (VV- GP, provided by J.L. Whitton) was generated and propagated as described [20]. For secondary challenges, mice were injected i.p. with 2 x 10^6 PFU.

Irradiation chimeras

To generate WT/IL-2Rα-deficient mixed bone marrow chimeras, recipient mice were given 900 rads using an analytical X-ray irradiator located in the mouse vivarium at the University of Utah. One day later, we harvested bone marrow from the femurs and tibias of WT and IL-2Rα-deficient donors. Following red blood cell lysis, bone marrow cells were incubated with biotinylated anti-CD3 antibodies (eBioscience, San Diego, CA), followed by incubation with anti-biotin magnetic beads (Miltenyi). $CD3^+$ cells were depleted by passage through a magnetic column according to the manufacturer's instructions (Miltenyi). CD3-depleted WT and IL-2Rα-deficient bone marrow cells were mixed 1:1 and injected i.v. into irradiated hosts. By using different combinations of congenic markers, we readily distinguished WT ($Ly5.1^+$), IL-2Rα-deficient ($Thy1.2^+$) and residual host ($Thy1.1^+$) T cells in the periphery of host mice 8-10 weeks later. Similar methods were used to generate P14 irradiation chimeras. P14 irradiation chimeras were generated with a 1:1 mix of WT or IL-2Rα-deficient P14 bone marrow with B6 bone marrow. P14 were harvested a minimum of 8 weeks postirradiation.

Cell suspensions and adoptive transfers

Splenocytes and lymph node cells were harvested at the indicated time points and re-suspended in RPMI 1640 supplemented with 10% FBS and antibiotics. Liver and lung lymphocytes were harvested by collagenase digestion as previously described [21]. Untouched $CD8^+$P14 T cells were isolated from the spleens and lymph nodes of WT or

IL-2Rα-deficient P14 bone marrow chimeras by incubation with a biotinylated antibody cocktail followed by anti-biotin magnetic beads and depletion on a magnetic column, per manufacturer's recommendations (Miltenyi). In addition, we added biotinylated CD44 antibody (eBioscience, San Diego, CA) to eliminate $CD44^{hi}$ "memory phenotype" P14. TCR transgenic T cell purity was assessed by staining with CD44, Vα2 and Vβ8.1 antibodies, followed by flow cytometric analysis. WT ($Thy1.1^+$) and IL-2Rα-deficient ($Thy1.1^+Thy1.2^+$) P14 were mixed 1:1 and co-injected i.v. into naïve B6 ($Thy1.2^+$) mice at the indicated doses one day prior to infection.

Peptide restimulation and intracellular cytokine staining

Splenocytes were re- suspended in RPMI 1640 containing 10% fetal bovine serum and supplemented with antibiotics and L-glutamine. Mice were re-stimulated with 0.1 μM H-$2D^b$-restricted peptide (gp_{33-41}) in the presence of Brefeldin A (1 μg/ml GolgiPlug). Cells were stained with cell surface antibodies, permeabilized and stained with cytokine antibodies (specific to IFNγ, TNFα and IL-2) using a kit per manufacturer's instructions (BDBiosciences, Mountain View, CA).

CTL assays

We utilized a FACS-based cytotoxicity assay as previously described [22]. EL4 cells were incubated with 0.1 μM gp_{33-41} peptide for 2 hours at 37° C. Cells were washed and incubated with FACS-sorted WT or IL-2Rα-deficient P14 CTLs for two hours at 37° C at effector to target ratios ranging from 3:1 to 0.1:1. We stained for expression of Annexin V (BDBiosciences) and measured the percent of Annexin V+ target cells by FACS. Specific killing was determined by comparison to killing of unloaded control targets cells.

Tetramer staining and analysis

The H-2D^b-restricted gp_{33-41} monomer was generated, biotinylated and tetramerized with streptavidin-conjugated allophycocyanin using described methods [23, 24], with modifications as described in protocols available on the NIH Tetramer Core Facility website (http://tetramer.yerkes.emory.edu/). Staining was performed at 4°C for 1 hour in FACS buffer (PBS with 2% fetal bovine serum and .02% sodium azide).

Antibodies and flow cytometry

Fluorescent dye-conjugated antibodies were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA) or BDBiosciences (Mountain View, CA) with the following specificities: CD8, Thy1.1, Thy1.2, CD45.1, Vα2, Vβ8.1, CD44, IL-7Rα, KLRG1, CD27, CD62L, CXCR3, CD43, CD122, CD25, Eomes, T-bet, Granzyme B, CD107a, TNFα, IL-2 and IFNγ. Cell surface antibody staining was done in PBS containing 2% FBS, and intracellular cytokine staining was performed as described above. For T-bet and Granzyme B staining, cells were permeabilized using the same buffers as for intracellular cytokine staining (BDBiosciences, Mountain View, CA). For Eomes, cells were permeabilized and stained using the same buffers as those used for the anti-FoxP3 antibody per the manufacturer's instructions (eBioscience, San Diego, CA). For CD107a, the antibody was mixed with re-suspended cells during peptide restimulation prior to intracellular cytokine staining. Multi-parameter (6-7 color) analysis of antibody-stained cells was performed on a FACSCanto II flow cytometer (BDBiosciences, Mountain View, CA) and results analyzed using FlowJo software (TreeStar). Cell sorting was with on a FACSVantage (BDBiosciences, Mountain View, CA) at the University of Utah FACS core facility.

Microarray

RNA was isolated using RNeasy kits (Qiagen) from FACS-sorted P14 cells (day 8 postinfection). Message was amplified and converted to Cy3 or Cy5-labeled cRNA using a commercially available kit per manufacturer's instructions (Agilent Technologies). Four biological duplicates from each group were hybridized to Agilent whole mouse genome microarrays. For each of the four replicates, dual hybridization was performed using RNA obtained from WT and IL-2Rα-deficient P14 isolated from the same animal. Results were normalized and analyzed for differences in log2 expression values using GeneSifter software (Geospiza, Seattle, WA). Microarray data has been submitted to the on-line depository GEO and conforms to all MIAME guidelines.

Results

Impaired accumulation and survival of $CD8^+$ end stage effector T cells in the absence of IL-2 signals

Our previous studies found that in the absence of IL-2 signals, developing $CD8^+$ memory T cells rapidly converted to a $CD62L^{hi}$ phenotype [15]. To characterize this finding further, we analyzed the responses of LCMV-specific P14 TCR transgenic T cells either expressing or lacking the IL-2Rα. IL-2Rα-deficiency results in the loss of $CD4^+CD25^+$ regulatory T cell function and multi-organ autoimmunity at a young age [25-27]. In order to mitigate non-specific effects this environment might have on T cell function, we sought to generate IL-2Rα-deficient P14 donors that lacked autoimmune side effects. We generated mixed bone marrow chimeras by transferring a 1:1 mix of wildtype (WT) B6 and either WT or IL-2Rα-deficient P14 bone marrow (BM) into lethally irradiated B6 hosts, as previously described [15]. Because the WT BM gave rise to a functional regulatory T cell population, the chimeras remained healthy with no signs of autoimmunity. Naïve ($CD44^{lo}$) WT and IL-2Rα-deficient P14 cells were harvested from

the chimeras and co-transferred into naïve B6 hosts, followed by LCMV infection. Because of their variable expression of Thy1 alleles, we simultaneously tracked WT P14 (Thy1.1$^+$) and IL-2Rα-deficient P14 (Thy1.1$^+$Thy1.2$^+$) responses in B6 hosts (Thy1.2$^+$) at various time points postinfection.

As previously observed [15], WT P14 responders expanded modestly (2-3-fold) better than IL-2Rα-deficient P14 responders by the peak of the response. Both populations also formed memory populations that persisted at stable levels throughout the course of our experiments (Fig. 2.1A). However, a slightly higher fraction of IL-2Rα-deficient P14 cells was lost during the contraction phase, as compared to the peak of the response (Fig. 2.1B). To determine the cause of this loss, we analyzed the differentiation of effector cell and memory precursor populations at the peak of the response and in the transition to memory. Recent studies have found that terminal effector phenotype CTL can be differentiated from memory precursor/memory cells based on variable expression of IL-7Rα and KLRG1 [3]. At the peak of the primary response (day 8), we observed 2-3-fold fewer KLRG1^{hi}IL7Rα^{lo} effector CTL among the IL-2Rα-deficient P14 responders, as compared to WT P14. WT P14 formed a population of detectable KLRG1^{hi} terminal effector phenotype CTL that slowly faded from the memory pool over the course of 4-6 months (referred to here as short-term effector memory cells). In contrast, IL-2Rα-deficient effector cells disappeared rapidly, comprising 10-20-fold lower levels in the spleen at day 42 postinfection (Fig. 2.2). WT and IL-2Rα-deficient P14 demonstrated no differences in the generation of KLRG1^{lo}IL7Rα^{hi} memory precursors at the peak of the response (day 8 postinfection). Numerical differences in the number of WT and IL-2Rα-deficient P14 could almost entirely be ascribed to deficiencies in the generation of terminal effector phenotype cells. Several other markers also confirmed the rapid disappearance of effector cells in the IL-2Rα-deficient P14 population. Besides their expression patterns of KLRG1 and IL-7Rα, differentiated effector populations were

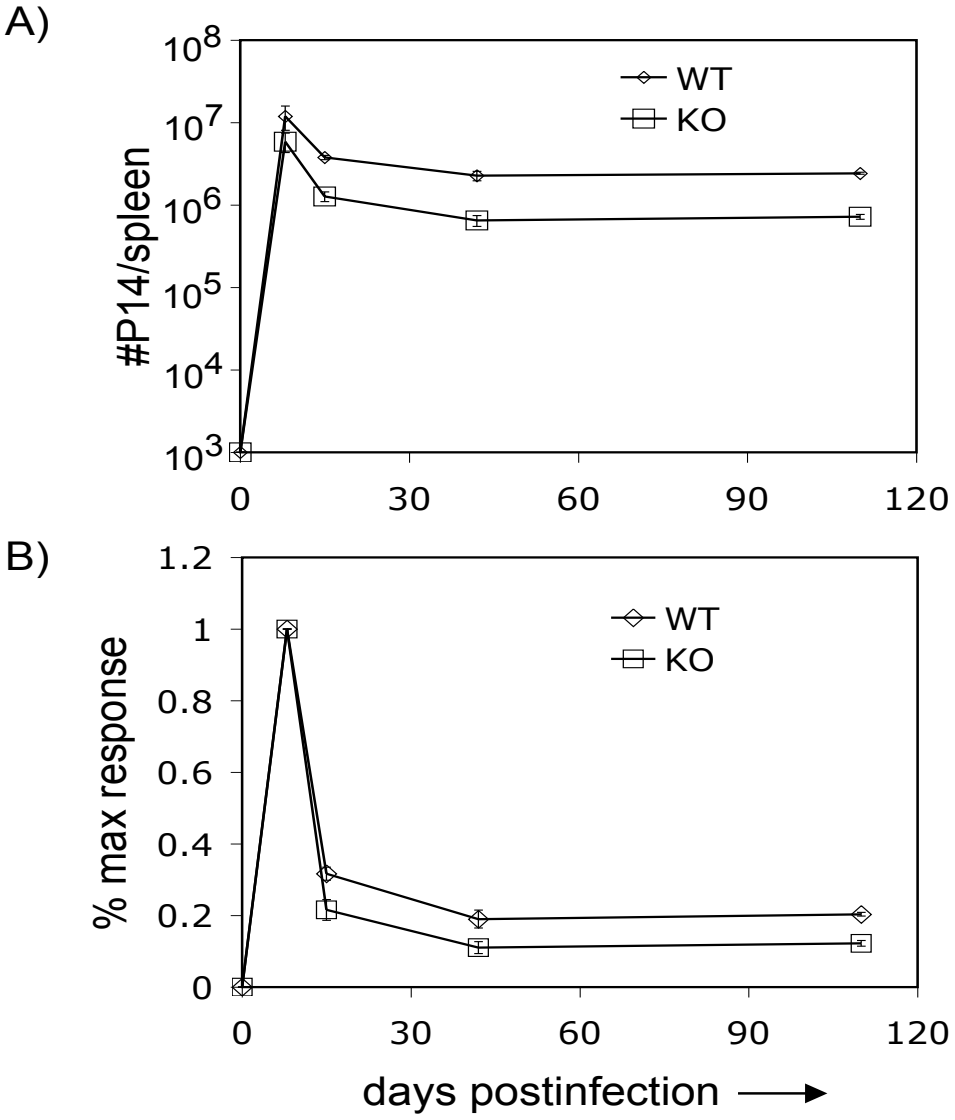

Figure 2.1 The magnitude of the CD8+ T cell response is modestly reduced in the absence of IL-2. Naïve (CD44-lo) WT and IL-2Rα-/- (KO) P14 were harvested from the spleens and lymph nodes of P14 BM Chimeras, mixed 1:1, and coinjected into naïve B6 mice 1 day prior to LCMV infection. A) The graphs display the total number of WT and IL-2Rα-/- P14 harvested from the spleen at indicated time points. B) The fraction of surviving P14 cells within each group as compared with the peak response (day 8). Error bars indicate the SEM (n=3-4 per time point, statistical significance determined by Student t test throughout the text). Results are representative of 5-6 independent experiments.

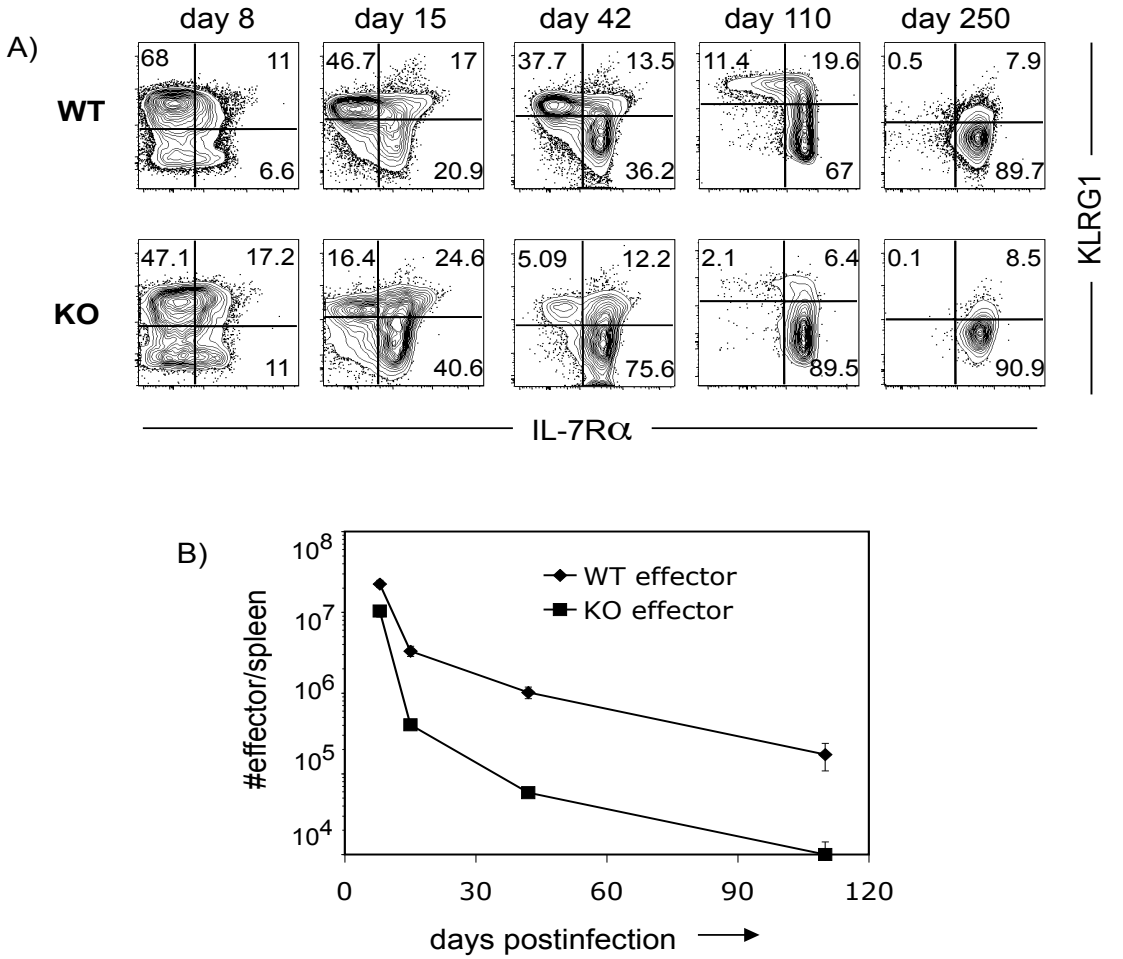

Figure 2.2 IL-2 drives the differentiation and survival of terminal effector phenotype and effector memory CD8+ T cells. Naïve (CD44-lo) WT and IL-2Rα-/- (KO) P14 were harvested from the spleens and lymph nodes of P14 BM Chimeras, mixed 1:1, and coinjected into naïve B6 mice 1 day prior to LCMV infection. A) Representative flow plots display the frequency of terminal effector phenotype P14 (KLRG1-hi, IL7Rα-lo) and memory precursor/memory P14 (KLRG1-lo, IL7Rα-hi) at the indicated time points post infection. B) Graphs show the total number of end-stage effector P14 (KLRG1-hi, IL7Rα-lo) at the indicated time points postinfection. Error bars indicate the SEM (n=3-4 per time point). Results are representative of 5-6 independent experiments.

$CD27^{lo}$, $CD62L^{lo}$, $CXCR3^{lo}$ and $CD43^{lo}$. Effector cells bearing these characteristics also disappeared rapidly in the absence of IL-2 signals in both the spleen and liver (Fig. 2.3 and Fig. 2.4).

Lack of IL-2 signals adversely impacts the function of effector CTLs

Because we observed modest but reproducible differences in the number of $CD8^+$ effector CTL at the peak of the response in the absence of IL-2 signals, we tested the hypothesis that IL-2 might also be important for optimal effector function. Previous studies in which CTL received little or no IL-2 signals were disrupted during in vitro activation followed by in vivo adoptive transfer suggested that IL-2 might play an important role in the development of effector function [28]. We focused on a time point at which the high affinity IL-2 receptor was expressed at high levels (day 5) as well as the peak of the effector response (day 8), at which time the high affinity IL-2R was no longer expressed (Fig. 2.5A).

Initially, we measured intracellular expression of the transcription factors T-bet and Eomes. These related T-box transcription factors have been implicated in the differentiation of effector T cells and in the acquisition of effector T cell function, such as IFNγ production and CTL activity, as well as in the differentiation of functionally and phenotypically normal $CD8^+$ memory T cells [3, 22, 29, 30]. We found no differences in expression of T-bet at either day 5 or day 8 postinfection. Conversely, IL-2Rα-deficient P14 demonstrated a reproducible 2-fold increase in the amount of Eomes at day 8 postinfection (Fig. 2.5A). These differences were not due to differences in the composition of each population, as direct comparison of end-stage effector and memory precursor populations revealed the same 2-fold disparity in Eomes expression (data not shown). While these differences are modest, they remain of interest given that similar

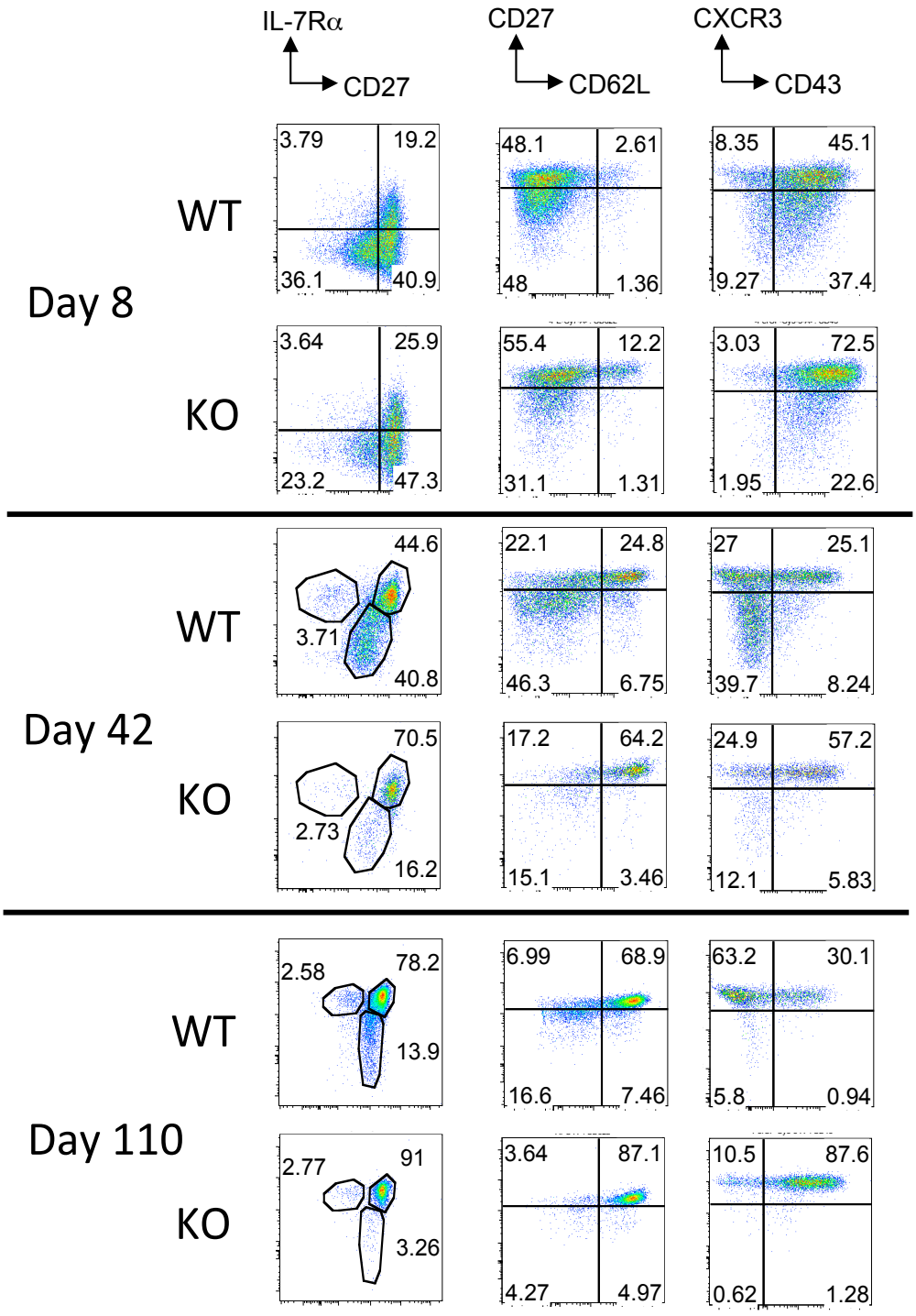

Figure 2.3 Terminal effector phenotype and effector memory CD8+ T cell differentiation and survival in the spleen is impaired in the absence of IL-2. Representative flow plots display cell surface expression of the indicated molecules by WT and IL-2Rα-/- (ko) P14 in the spleen at various time points postinfection with LCMV.

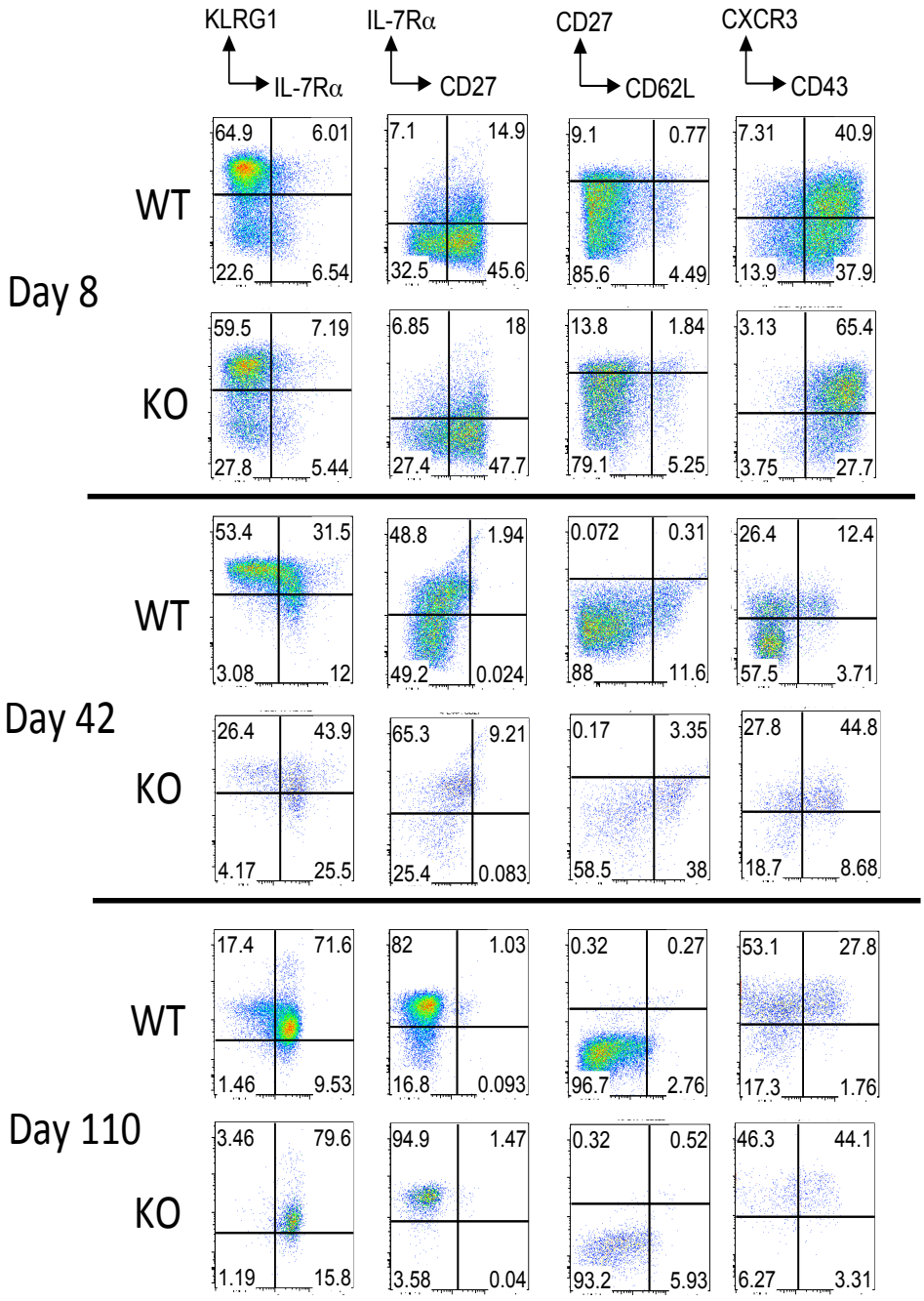

Figure 2.4 Terminal effector phenotype and effector memory CD8+ T cell differentiation and survival in the liver is impaired in the absence of IL-2. Representative flow plots display cell surface expression of the indicated molecules by WT and IL-2Rα-/- (ko) P14 in the liver at various time points postinfection with LCMV.

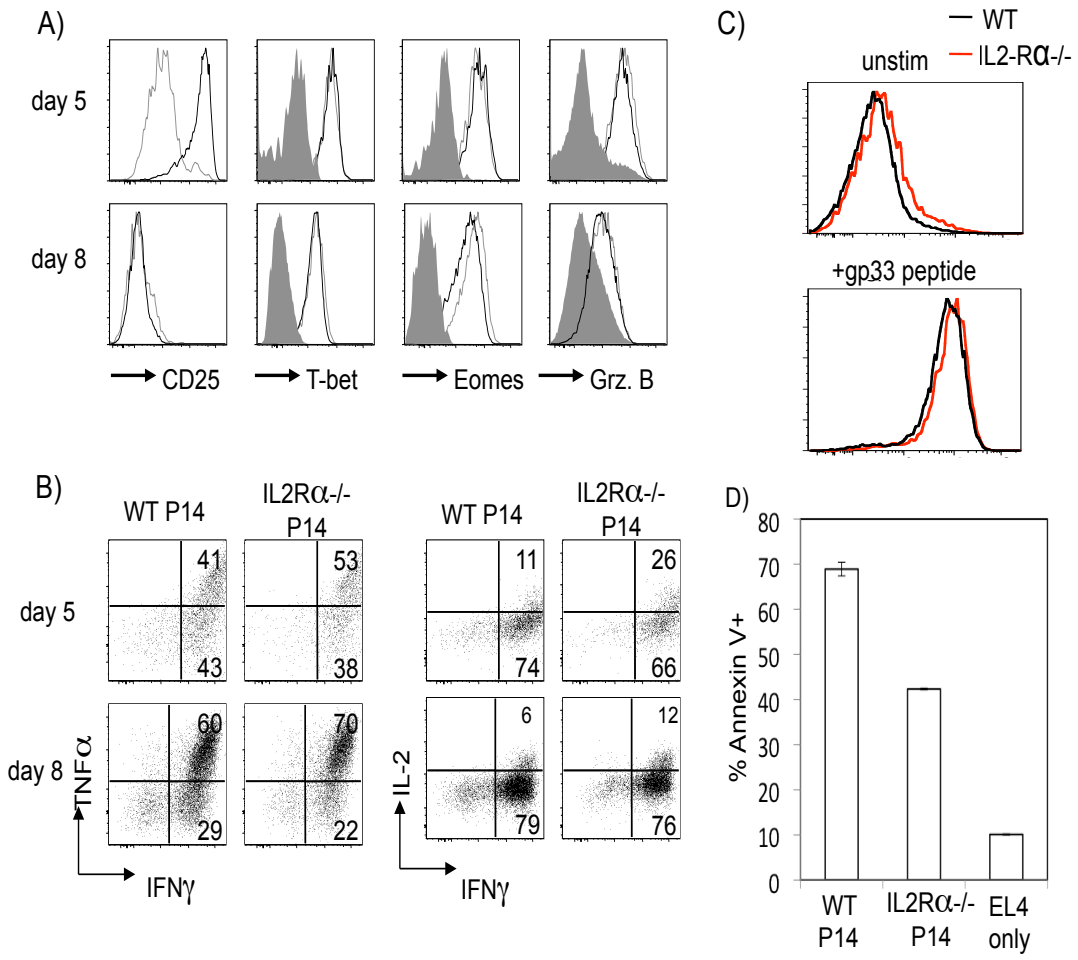

Figure 2.5 IL-2Rα-deficient P14 CTLs have modestly decreased cytolytic activity. Effector WT and IL-2Rα-/- P14 were assessed for expression of effector molecules and function. A) WT (black line) and IL-2Rα-/- (gray line) P14 were tested for cell surface expression of CD25 and intracellular expression of T-bet, Eomes and Granzyme B on days 5 and 8 postinfection, as indicated. Gray filled histograms are isotype controls. B) We assessed production IFNγ, TNFα and IL-2 by WT and IL-2Rα-/- P14 as indicated in representative plots following 4 hour ex vivo restimulation with gp_{33-41} peptide in the presence of Brefeldin A. C) Representative flow plots indicate CD107a surface expression as a measure of degranulation during a 4 hour ex vivo gp_{33-41} restimulation of WT and IL-2Rα-/- P14 at day 8 postinfection with LCMV. D) gp_{33-41} peptide loaded EL4 cells were incubated with WT or IL-2Rα-/- P14 CTL FACS-sorted from spleens at day 8 postinfection. CTL induced apoptosis was measured by staining with Annexin V 2 hours later. Error bars represent the SEM (n=3-4/group) and differences between WT and IL-2Rα-/- (KO) P14 are statistically significant ($p<0.01$).

differences in Eomes expression in mice with a single functional allele impacts $CD8^+$ T cell differentiation and effector function [29]. Nevertheless, these results are inconsistent with an obligate role for IL-2 in the acquisition of effector function, given that Eomes expression was higher in the absence of IL-2. Furthermore, they suggest that Eomes expression is influenced indirectly by IL-2, as no expression differences were seen at day 5 when WT P14 responders are still actively receiving IL-2 signals.

IL-2Rα-deficient CTL expressed Granzyme B and de-granulated and produced cytokines upon re-stimulation (Fig. 2.5A, B, C). However, IL-2Rα-deficient CTL demonstrated a modest decrease in CTL activity at day 8 postinfection (Fig. 2.5D). To assess effector CTL development at this time point, we analyzed RNA expression by WT and IL-2Rα- deficient P14 CTL by microarray. We observed significant up-regulation of effector molecules involved in cytolysis and effector differentiation in WT CTL, including Granzymes and Perforin, as well as an increase in T-bet (Table 2.1). While Blimp-1 was not significantly up-regulated in WT cells, Bcl-6 was significantly up-regulated in IL-2Rα- deficient CTL (Table 2.2). Because Blimp-1, which has recently been shown to promote effector and memory CTL differentiation [31, 32], is a potential repressor of Bcl-6 expression in T cells [33], this finding leaves open the possibility that its transcriptional activity is posttranscriptionally or posttranslationally reduced in the absence of IL-2 signals, despite no changes in mRNA expression. A variety of NK receptors, likely indicators of CTL differentiation [3], also demonstrate increased expression in WT CTL. IL- 2Rα-deficient CTL expressed higher levels of IL-2 and TNFα as well as receptors that mediate trafficking to and within secondary lymphoid organs (CCR7 and CXCR5)(Table 2.2). These findings were predicted by cell surface staining and again indicate a skewing away from a differentiated effector phenotype. While we observed differences in expression of several pro and antiapoptotic mediators, no clear pattern emerged to explain the failure of IL-2Rα-deficient effector CTL to survive

Table 2.1 Genes with increased expression in WT P14 compared to IL-2Rα-/- P14 on day 8 postinfection with LCMV. All fold differences are significant ($p<0.05$, n=4 per group) (*no significant difference). diff, differentiation

Name	fold up WT	function
Granzyme A	3.3	cytotoxicity
Granzyme B	1.8	cytotoxicity
Granzyme E	2.4	cytotoxicity
Perforin	1.4	cytotoxicity
Klre1	1.9	NK/CTL diff. marker
Klrb1b	2.3	NK/CTL diff. marker
Klrk1	1.7	NK/CTL diff. marker
Klrg1*	1.2	NK/CTL diff. marker
Klrc2	1.5	NK/CTL diff. marker
Klra3	1.5	NK/CTL diff. marker
Klra7	1.8	NK/CTL diff. marker
2B4	1.7	NK/CTL diff. marker
IL12Rb2	1.5	cytokine receptor
Tbet	1.5	CTL differentiation
Blimp-1*	1.2	CTL differentiation
Cdca2	1.5	cell cycle
Ki-67	1.5	cell cycle
Cdc25b	1.6	cell cycle
Cyclin A2	1.7	cell cycle
Cdc451	1.7	cell cycle
Cdc7	1.8	cell cycle
Gspt2	1.8	cell cycle
Cdca5	1.8	cell cycle
Cdc23	1.9	cell cycle
Rb1	1.8	cell cycle arrest
Cdkn1a	1.9	cell cycle arrest
Bcl-XL	1.5	survival
FasL	1.5	apoptosis
Caspase 3	1.6	apoptosis/proliferation
c-Flip	1.6	apoptosis/proliferation
RIP-1	1.6	apoptosis/proliferation
Faf-1	1.5	apoptosis
Jak-1	1.6	Cytokine signaling
NFAT4	1.7	Transcription Factor
PLCγ-1	1.7	TCR signaling
Socs2	3.7	Neg. regulation of JAK/STAT

Table 2.2 Genes with increased expression in IL-2Rα-/- (KO) P14 compared to WT P14 on day 8 postinfection with LCMV. All fold differences are significant ($p<0.05$, n=4 per group).

Name	fold up IL-2Rα-/-	function
Bcl6	1.9	memory differentiation
IL-2	2.3	cytokine
TNFa	1.5	cytokine
IL-10	1.9	cytokine
IL-17F	1.7	cytokine
CXCR5	5.4	SLO trafficking
CCR7	3.3	SLO trafficking
CTLA4	1.5	neg. costimulation
CD40	2.7	costimulation
CD44	1.6	activation marker
TCF-1	2.2	CTL diff. arrest
LEF-1	1.3	CTL diff. arrest
Bcl2	1.8	Survival
Fas	2.1	apoptosis
Bim	1.7	apoptosis
Egr1	2.3	induction of NFAT
Egr4	3.5	induction of NFAT
c-jun	1.8	gene activation
Xcl1	2.7	T cell/DC interactions
CD30L	3.5	activation
CD9	2.2	adhesion
GATA-2	1.6	fate determination

following antigen clearance. However, WT P14 CTL demonstrated enhanced expression of a variety of cell cycle participants, indicating that IL-2 may drive cell division later in the primary response (Table 2.1). This finding corresponds to previous observations by others [13, 34] and our own finding that WT responders demonstrate enhanced expansion between days 5 and 8 postinfection [15]. Overall, while many of the gene expression differences are individually modest, they collectively support a role for IL-2 in driving the differentiation and enhancing the function of primary CTL. One possible interpretation of these results is that differences in cytolytic function (Fig. 2.5D) and expression of CTL differentiation markers and cytolytic molecules (Table 2.1) reflect differences in the generation of terminal effector phenotype CTL in the absence of IL-2 (Fig. 2.2), and future studies will be needed to directly compare the differentiation status of purified terminal effector phenotype and memory precursor $CD8^+$ cell populations in the presence or absence of IL-2 signals.

Poor differentiation of secondary effector T cells
in the absence of IL-2

Because $CD8^+$ memory T cells generated in the absence of IL-2 signals mount poor secondary responses, we assessed their ability to become secondary effector cells. To assess polyclonal endogenous recall responses, we generated mixed bone marrow chimeras using a 1:1 mix of bone marrow from WT and IL-2Rα-deficient donors. At 8-10 weeks posttransplant, mice were challenged with LCMV. As measured by MHC Class I tetramers (Fig. 2.6A) and the frequency of IFNγ producing cells following peptide restimulation, even in the absence of IL-2 signals $CD8^+$ T cells generated robust primary responses and long-lived memory populations similar to that of wildtype responders. Mice were rechallenged with either a recombinant *Listeria monocytogenes* expressing the LCMV glycoprotein (Lm-gp33) or a recombinant vaccinia virus expressing the LCMV

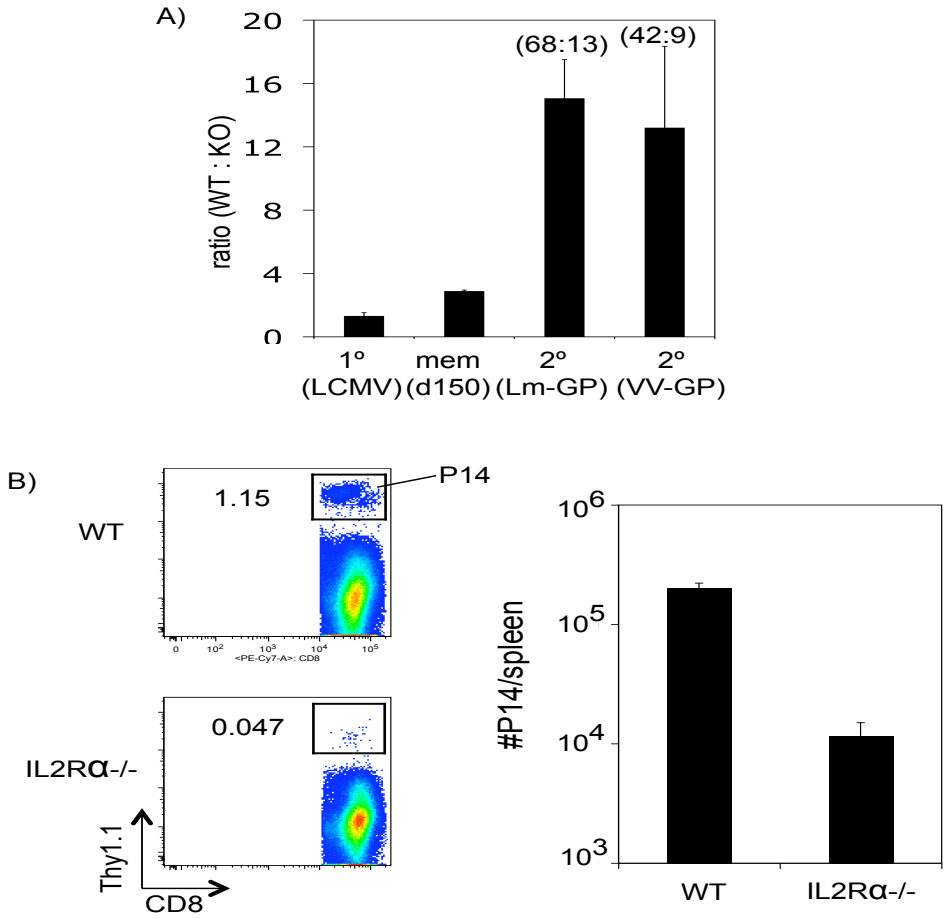

Figure 2.6 IL-2Rα-deficient memory CD8+ T cells fail to accumulate upon secondary challenge. A) We generated mixed BM chimeras using WT and IL-2Rα-/- donor BM cells in equal number. At 10 weeks post transplant, we infected chimeras with LCMV and assessed CD8+ T cell responses in the spleen using D^b-gp33-41 tetramer at the peak of the primary response (day 8 postinfection), during memory maintenance (day 150 postinfection), or at the peak of the secondary response (day 5 post rechallenge with Lm-gp33 or VV-gp). The bar graph indicates the ratio of WT and IL-2Rα-/- tetramer-binding responders at the indicated time points. B) WT and IL-2Rα-/- P14 (500 of each) were co-transferred into B6 mice, followed by infection with LCMV one day later. WT and IL-2Rα-/- memory P14 cells (>300 days postinfection) were FACS-purified from the spleen and transferred separately (10,000 of each) into naïve B6 hosts. Secondary hosts were challenged with LCMV and recall responses assessed in the spleen 7 days later. Flow plots show the frequency of WT or IL-2Rα-/- P14 at the peak of rechallenge, and bar graphs indicate the fold expansion. Results are representative of 3-4 separate experiments. Error bars indicate the SEM (n=3-4/group), and differences in fold expansion are statistically significant, $p<0.05$.

glycoprotein (VV-GP) at 150 days postinfection. As previously observed [15], IL-2Rα-deficient $CD8^+$ memory T cells demonstrated a 10-15-fold deficit in their ability to generate secondary responses, as compared to WT. Furthermore, poor recall responses were not due to competition with WT memory CTL. Similar differences were seen when FACS-purified WT and IL-2Ra-deficient memory P14 were transferred into separate naïve B6 hosts prior to rechallenge (Fig 2.6B).

A closer analysis of the tetramer binding cells at the peak (day 5) of the secondary response revealed that IL-2 signals were required for the generation of secondary effector CTL ($KLRG1^{hi}IL\text{-}7R\alpha^{lo}$) (Fig. 2.7A, C). Furthermore, secondary responders induced in the absence of IL-2 remained CD62Lhi (Fig. 2.7A), $CD27^{hi}$ and $CXCR3^{hi}$ (Fig. 2.7B), all characteristics of memory cells, not effector cells. These defects were present but modest at the peak of the primary response and greatly exacerbated upon secondary challenge (Fig. 2.7A,B). To assess function, we restimulated splenocytes ex vivo both before and after rechallenge. Prior to rechallenge, both wildtype and IL-2Rα-deficient memory populations primarily consisted of cells capable of simultaneously producing IFNγ and TNFα (double producers), or IFNγ, TNFα and IL-2 (triple producers), with few cells only able to produce IFNγ (single producers). Following rechallenge and the development of secondary effectors, the cytokine-producing profile of WT responders shifted dramatically towards single or double producers, with few triple producers. Conversely, the cytokine-producing profile of IL-2Rα-deficient secondary responders was largely unchanged, again reflecting an inability to generate large numbers of secondary effector CTL (Fig. 2.7D).

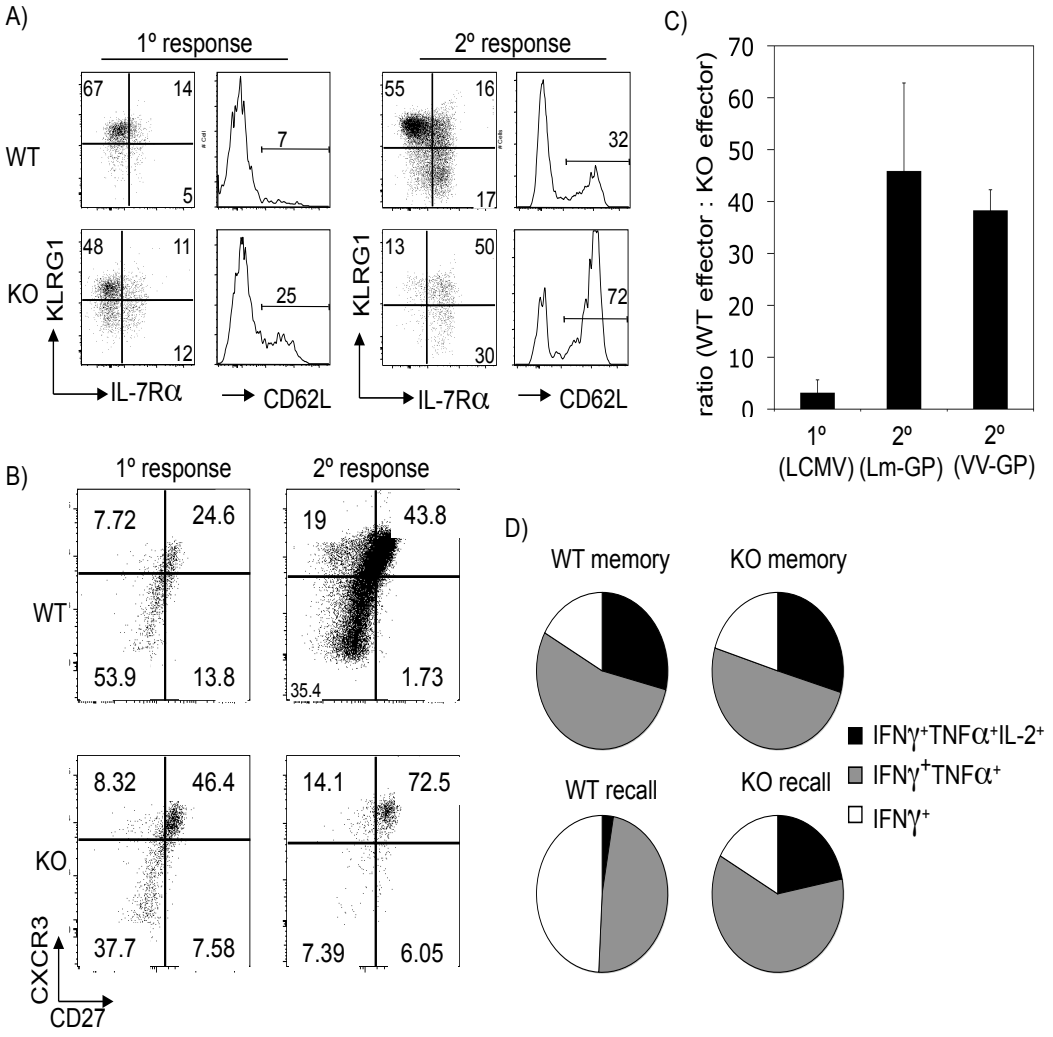

Figure 2.7 IL-2Rα-deficient CD8+ memory T cells fail to differentiate into secondary effector CTLs upon rechallenge. WT and IL2Rα-/- Bone Marrow chimeras were generated, infected, and rechallenged as described in Figure 2.6. A) Representative flow plots, gated on tetramer binding CD8+ T cells, indicate the frequency of terminal effector phenotype (KLRG1-hi, IL7Rα-lo, or CD62L-lo) cells among WT or IL-2Rα-/-(KO) responders at the indicated time points. B) Representative flow plots show CXCR3 and CD27 expression at the indicated time points. C) The bar graphs indicate the ratio of WT to IL-2Rα-/- tetramer binding CD8+ T cells that display a KLRG1-hi, IL7Rα-lo terminal effector phenotype at the peak of the primary or secondary response with the indicated infections. Error bars indicate the SEM (n=3-4/group, $p<0.05$ when comparing ratios at primary or secondary response). D) After 4 hours of ex vivo gp33-41 peptide restimulation of splenocytes, we assessed the ability of WT and IL-2Rα-/- responders to make cytokines at either day 150 (memory) or day 5 post rechallenge with Lm-gp33 (recall).

IL-2 promotes the differentiation of CD8⁺ effector memory T cells

Our prior studies confirmed that CD8⁺ memory T cells generated in the absence of IL-2 signals quickly converted to a CD62L^{hi} central memory phenotype [15]. However, because in the present study we observed in the absence of IL-2 signals a rapid loss of KLRG1^{hi}IL-7Rα^{lo} effector cells that are also CD62L^{lo}, we considered the possibility that our prior observations simply reflected a loss of this population and not a role for IL-2 in the generation of bona fide effector memory T cells. To test this possibility we assessed CD62L expression by IL-7Rα^{hi} memory T cells as a measure of true effector memory T cell differentiation and survival. We found that even this population demonstrated a rapid loss of CD62L^{lo} effector memory T cells in the spleen following acute LCMV infection in the absence of IL-2 signals (Fig. 2.8A). Furthermore, we found that WT tissue-residing memory P14 in the liver demonstrated a selective advantage over time as compared to IL-2Rα-deficient memory P14. This survival advantage was intermediate in the spleen and not observed in the lymph nodes (Fig. 2.8B, C). We therefore concluded that IL-2 played a central role in the differentiation of effector memory T cells in both the spleen and peripheral sites of infection.

In all, these data indicate that IL-2 influences a wide spectrum of effector differentiation, from end-stage effector cells at the peak of the response to both short lived and long lived effector memory CTL in secondary lymphoid tissues and peripheral sites of infection. Numerically, the differentiation of central memory T cells appears to be independent of IL-2, as we observe roughly similar numbers of central memory phenotype WT and IL-2Rα-deficient CTL at early memory points (data not shown). Because IL-2Rα-deficient memory CTL at early memory time points are largely central memory phenotype already, the overall number of central memory cells remains stable

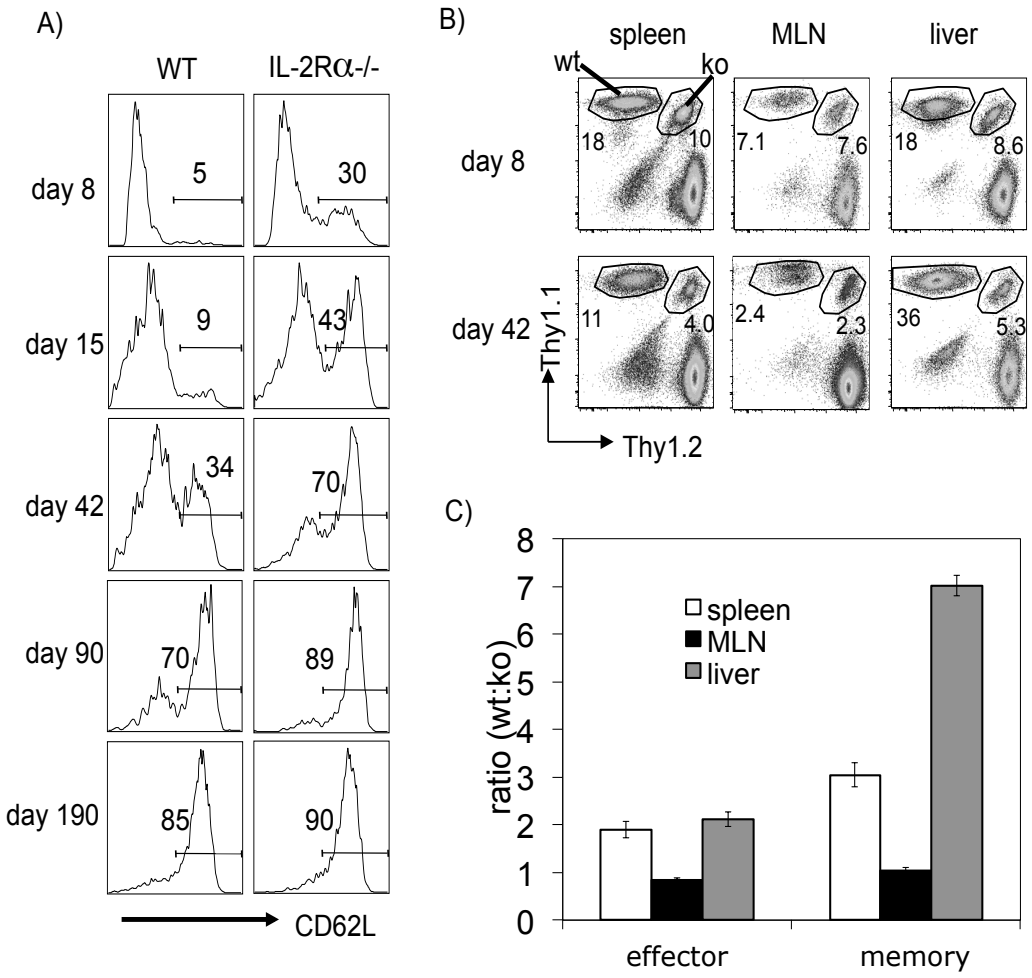

Figure 2.8 IL-2 drives the differentiation of long lived tissue residing effector memory CTLs. We transferred 500 WT and IL-2Rα-/- P14 cells prior to LCMV infection. A) Representative flow plots indicate the frequency of central memory (CD62L-hi) phenotype cells in the spleen at the indicated time points after LCMV infection. All plots are gated on the memory precursor/memory population (KLRG1-lo, IL-7Ra-hi). B) Representative flow plots indicate the relative frequencies of WT and IL-2Rα-/- P14 (ko) in the spleen, mesenteric lymph nodes (MLN) and liver at days 8 and 42 postinfection. C) The bar graph displays the ratio of WT to IL-Rα-/- responders at day 8 (effector) and day 42 (memory) postinfection with LCMV in the indicated tissues. Error bars indicate the SEM (n=3) and the results are representative of four separate experiments. The ratio increase in the liver at day 42 is statistically significant as compared to the spleen and lymph nodes ($p<0.01$).

throughout memory maintenance. On the other hand, at early memory time points the WT memory population is largely composed of short-term and long-term effector memory cells. Over time, the memory population remains stable but eventually converts to a central memory phenotype, whether due to conversion of existing effector memory cells to central memory cells [32]or replacement of effector memory cells with central memory cells due to homeostatic mechanisms[33]. While the end result at late memory time points is a 3-fold difference in the number of central memory cells (Fig. 2.1), our results suggest that IL-2 mainly plays a role in effector and effector memory CTL differentiation and that the defect in central memory cells is more precisely one of secondary effector differentiation.

Discussion

Our understanding of the role of IL-2 during in vivo immune responses has undergone changes and revisions over the years. Although it was originally thought to be required for T cell expansion, we find robust $CD8^+$ T cell expansion even in the complete absence of high affinity IL-2 signals. However, our findings suggest that IL-2 plays a unique and important role as a fate determination and differentiation signal for activated T cells in vivo. Here again, the role of IL-2 is complex. While IL-2 plays a role in promoting the emergence and function of effector CTLs during the primary response, its impact is particularly pronounced in the rapid disappearance of this population during the contraction phase. Expression of the high affinity IL-2 receptor generally corresponds with bursts of IL-2 production in vivo, with the notable exception of the regulatory T cell subset. We observe little to no expression of IL-2Rα at day 8 postinfection or beyond, indicating that the high affinity IL-2R signal is confined to the primary T cell response and expansion phase. We therefore conclude that IL-2 signals during priming influence the generation of effector CTL during primary expansion but

more particularly their persistence once the virus is cleared. These findings are consistent with previous results demonstrating that IL- 2 signals during the first week of infection promote the subsequent survival of IL-7Rα^{lo} and CD62L^{lo} responders during the contraction phase [15]. Two recent reports have also demonstrated a role for IL-2 in driving effector CTL differentiation. These studies found that effector CTL differentiation was influenced by the concentration of IL-2 following activation[35] or by the length of time activated CTLs were able to incorporate high affinity IL-2 signals [36]. Together with our current report, these studies demonstrate a nonredundant role for IL-2 in enhancing effector CTL differentiation, survival and function. Our report further demonstrates that in the complete absence of high affinity IL-2 signals, secondary effector CTL differentiation is dramatically impaired. One intriguing possibility is that while strong IL-2 signals enhance effector differentiation, some IL-2 signals are required in order for memory precursor effector cells to maintain, perhaps through epigenetic changes, their ability to access the effector differentiation transcriptional program. Thus memory cells generated in the absence of IL-2 signals would be largely unable to enter an effector differentiation pathway upon reactivation. Future studies are needed to identify epigenetic changes as well as changes in transcriptional activity that are influenced by IL-2 signals in differentiating CTL in vivo.

Of particular interest is our finding that the generation and/or survival of secondary effector CTL is largely disabled in the absence of IL-2 signals. Our prior findings have demonstrated that IL-2Rα-deficient CD8^{+} memory T cells divide rapidly upon rechallenge but fail to accumulate as compared to wildtype memory cells [15]. We find here that despite their rapid division, almost no secondary effector CTL emerge in the absence of IL- 2 signals. While there is some evidence of effector differentiation, including up-regulation of KLRG1, secondary effector cells rapidly disappear from the response, suggesting that IL-2 provides a differentiation signal to activated T cells that

enables or potentiates entry into an effector/effector memory lineage. Prior studies have shown that secondary effector and memory T cells skew more strongly to the effector and/or effector memory lineage, maintaining low levels of CD62L over long periods of time as compared to primary memory T cells[37].

We propose that IL-2 provides a differentiation signal that enables entry into and survival within the effector "program". This may include epigenetic imprinting during the primary response that enable or potentiate effector differentiation upon subsequent encounters with antigen. Because $CD8^+$ memory T cells are prone to become highly differentiated secondary effector/effector memory cells upon secondary activation, the absence of an IL-2 driven effector differentiation signal during the primary response may specifically and adversely impact the generation of highly differentiated secondary effector CTL. In this sense, IL-2 may be most appropriately described as an effector differentiation factor rather than a memory differentiation factor. While it may not be required for the selection of memory populations during the primary response, its role in driving effector differentiation is a key step in conferring the ability of memory T cells that do emerge to differentiate into effector cells upon secondary engagement with antigen.

The molecular and transcriptional nature of the IL-2-driven effector differentiation program remains unknown. Several transcription factors have been implicated in the differentiation of $CD8^+$ effector T cells. Of particular interest are the Tbox transcription factors T-bet and Eomesodermin (Eomes). T-bet and Eomes drive effector differentiation and are regulated in response to inflammatory signals such as IL-12 or Type I IFNs [3, 37, 38]. Eomes impacts the differentiation and survival of $CD8^+$ effector T cells by influencing the expression of effector molecules such as IFNγ and CD122 [29]. Another molecule of interest is the transcriptional repressor Blimp-1. While past studies have focused on the role of Blimp-1 in plasma cell differentiation, recent studies suggest

an important role for this molecule in CD8+ effector differentiation [39, 40] during acute viral infection and $CD8^+$ T cell exhaustion [41] during chronic viral infection. Blimp-1 induction during in vitro T cell activation is dependent on IL-2 and forms a feedback loop to inhibit IL-2 production[42]. Additionally, similar to responding CD8 T cells that do not receive IL-2 signals, Blimp- 1 deficient $CD8^+$ T cells also show a defect in effector and effector memory differentiation following acute infection [39, 40], as well as a reduced ability of Blimp-1 deficient memory cells to respond to rechallenge [39].

The levels of T-bet and Eomes protein expression, as well as Blimp-1 mRNA expression, were not reduced in the absence of IL-2 signals. Although we do not find an obligate role for IL-2 in inducing expression of any of these transcription factors during the in vivo response to viral infection, we do not rule out a role for IL-2 in controlling, directly or indirectly, their transcriptional activity. Although expression of the transcription factors T-bet, Eomes, and Blimp-1 are associated with effector CTL differentiation, less is known about how these transcription factors function and are regulated. For example, there may be activating and/or repressive binding partners and/or modifications affecting their activity. Thus, although our data shows that IL-2 is not obligatory for induction of expression of these factors, there are several ways in which IL-2 signals could result in alterations to transcriptional activity and, ultimately, the ability of a responding $CD8^+$ T cell to undergo effector differentiation. Furthermore, even modest differences (<2-fold) in expression and/or activity of some of these transcription factors may have a profound impact on T cell differentiation and function. Future studies will be needed to precisely elucidate the combined role of these transcription factors and others (such as the Blimp-1 repressor Bcl-6[43, 44]) in the differentiation of $CD8^+$ primary and secondary effector and effector memory T cells, along with the impact of inflammatory mediators and cytokines such as IL-2 on their activity.

IL-2 is used as an immunotherapy in situations where the inflammatory burst is comparatively minimal, such as for antitumor immune responses [45]. IL-2 also appears to play a significant role in maintaining effector/memory responses during chronic infections [46], indicating that the long-term ability to respond to antigen may require IL-2. It is in this way, perhaps, that in vivo responses reflect the need for IL-2 in promoting the establishment and maintenance of T cell lines in vitro. We anticipate that a more detailed understanding of the role of IL-2 in the differentiation and function of antigen-activated T cells will greatly enhance our understanding of memory T cell biology. In particular, defining its role will aid in a variety of therapeutic strategies aimed at manipulating the T cell response. These include vaccination, immunotherapeutic or immunomodulatory strategies aimed at boosting the immune response, tumor eradication by immune cells, and strategies for which immunosuppression is desirable, such as for prevention of autoimmune responses or transplant rejection.

References

1. Kaech, S.M. and E.J. Wherry, *Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection.* Immunity, 2007. **27**(3): p. 393-405.

2. Williams, M.A. and M.J. Bevan, *Effector and memory CTL differentiation.* Annual review of immunology, 2007. **25**: p. 171-92.

3. Joshi, N.S., et al., *Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor.* Immunity, 2007. **27**(2): p. 281-95.

4. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells.* Nature immunology, 2003. **4**(12): p. 1191-8.

5. Bevan, M.J., *Helping the CD8(+) T-cell response.* Nature reviews. Immunology, 2004. **4**(8): p. 595-602.

6. Janssen, E.M., et al., *CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes.* Nature, 2003. **421**(6925): p. 852-6.

7. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.

8. Sun, J.C. and M.J. Bevan, *Defective CD8 T cell memory following acute infection without CD4 T cell help.* Science, 2003. **300**(5617): p. 339-42.

9. Janssen, E.M., et al., *CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death.* Nature, 2005. **434**(7029): p. 88-93.

10. Chang, J.T., et al., *Asymmetric T lymphocyte division in the initiation of adaptive immune responses.* Science, 2007. **315**(5819): p. 1687-91.

11. Reiner, S.L., F. Sallusto, and A. Lanzavecchia, *Division of labor with a workforce of one: challenges in specifying effector and memory T cell fate.* Science, 2007. **317**(5838): p. 622-5.

12. D'Souza, W.N. and S.M. Hedrick, *Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program.* Journal of immunology, 2006. **177**(2): p. 777-81.

13. D'Souza, W.N. and L. Lefrancois, *IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion.* Journal of immunology, 2003. **171**(11): p. 5727-35.

14. Yu, A., et al., *Efficient induction of primary and secondary T cell-dependent immune responses in vivo in the absence of functional IL-2 and IL-15 receptors.* Journal of immunology, 2003. **170**(1): p. 236-42.

15. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells.* Nature, 2006. **441**(7095): p. 890-3.

16. Ahmed, R., et al., *Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence.* The Journal of experimental medicine, 1984. **160**(2): p. 521-40.

17. Shen, H., et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(9): p. 3987-91.

18. Slifka, M.K., et al., *Antiviral cytotoxic T-cell memory by vaccination with recombinant Listeria monocytogenes.* Journal of virology, 1996. **70**(5): p. 2902-10.

19. Williams, M.A., E.V. Ravkov, and M.J. Bevan, *Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory.* Immunity, 2008. **28**(4): p. 533-45.

20. Whitton, J.L., P.J. Southern, and M.B. Oldstone, *Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus.* Virology, 1988. **162**(2): p. 321-7.

21. Williams, M.A. and M.J. Bevan, *Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells.* Journal of immunology, 2004. **173**(11): p. 6694-702.

22. Cruz-Guilloty, F., et al., *Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs.* The Journal of experimental medicine, 2009. **206**(1): p. 51-9.

23. Altman, J.D., et al., *Phenotypic analysis of antigen-specific T lymphocytes.* Science, 1996. **274**(5284): p. 94-6.

24. Murali-Krishna, K., et al., *Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection.* Immunity, 1998. **8**(2): p. 177-87.

25. Fontenot, J.D., et al., *A function for interleukin 2 in Foxp3-expressing regulatory T cells.* Nature immunology, 2005. **6**(11): p. 1142-51.

26. Malek, T.R. and A.L. Bayer, *Tolerance, not immunity, crucially depends on IL-2.* Nature reviews. Immunology, 2004. **4**(9): p. 665-74.

27. Setoguchi, R., et al., *Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization.* The Journal of experimental medicine, 2005. **201**(5): p. 723-35.

28. Manjunath, N., et al., *Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes.* The Journal of clinical investigation, 2001. **108**(6): p. 871-8.

29. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin.* Nature immunology, 2005. **6**(12): p. 1236-44.

30. Pearce, E.L., et al., *Control of effector CD8+ T cell function by the transcription factor Eomesodermin.* Science, 2003. **302**(5647): p. 1041-3.

31. Obar, J.J., et al., *CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses.*

Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(1): p. 193-8.

32. Sarkar, S., et al., *Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation.* Journal of immunology, 2007. **179**(10): p. 6704-14.

33. Marzo, A.L., et al., *Initial T cell frequency dictates memory CD8+ T cell lineage commitment.* Nature immunology, 2005. **6**(8): p. 793-9.

34. Rubinstein, M.P., et al., *IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response.* Blood, 2008. **112**(9): p. 3704-12.

35. Pipkin, M.E., et al., *Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells.* Immunity, 2010. **32**(1): p. 79-90.

36. Kalia, V., et al., *Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo.* Immunity, 2010. **32**(1): p. 91-103.

37. Jabbari, A. and J.T. Harty, *Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype.* The Journal of experimental medicine, 2006. **203**(4): p. 919-32.

38. Agarwal, P., et al., *Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory.* Journal of immunology, 2009. **183**(3): p. 1695-704.

39. Kallies, A., et al., *Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses.* Immunity, 2009. **31**(2): p. 283-95.

40. Rutishauser, R.L., et al., *Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties.* Immunity, 2009. **31**(2): p. 296-308.

41. Shin, H., et al., *A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection.* Immunity, 2009. **31**(2): p. 309-20.

42. Gong, D. and T.R. Malek, *Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production.* Journal of immunology, 2007. **178**(1): p. 242-52.

43. Martins, G. and K. Calame, *Regulation and functions of Blimp-1 in T and B lymphocytes.* Annual review of immunology, 2008. **26**: p. 133-69.

44. Shaffer, A.L., et al., *BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control.* Immunity, 2000. **13**(2): p. 199-212.

45. Margolin, K., *Cytokine therapy in cancer.* Expert opinion on biological therapy, 2008. **8**(10): p. 1495-505.

46. Bachmann, M.F., et al., *Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections.* European journal of immunology, 2007. **37**(6): p. 1502-12.

CHAPTER 3

DISTINCT ROLES FOR THE CYTOKINES IL-2 AND IL-15 IN THE DIFFERENTIATION AND SURVIVAL OF EFFECTOR AND MEMORY $CD8^+$ T CELLS[*]

 Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells.* Journal of Immunology, 2010. 184(12): p.6719-30.

Introduction

Although IL-2 plays an important role in $CD8^+$ secondary effector T cell differentiation following secondary challenge, we remained puzzled by our observation that IL-2 played a much more modest role in driving robust expansion during the primary response. One likely explanation is that during in vivo infection, other growth and inflammatory factors compensate for the absence of IL-2.

We hypothesized that related cytokines may compensate for the lack of IL-2 signals during acute infection. IL-15 was an obvious initial candidate. IL-2 and IL-15 belong to a family of cytokines utilizing the common gamma chain (γ_c) as a component of their receptors. Among this family, IL-2 and IL-15 are particularly related due to sharing the β (CD122) and γ_c (CD132) chains of their heterotrimeric receptor. Therefore, IL-2 and IL-15 promote apparently distinct biological outcomes while utilizing similar JAK/STAT and protein tyrosine kinase (PTK) pathways [1, 2]. Because signals through both the IL-2R and the IL-15R are delivered by the β and γ_c chains, one possibility is that the biological effects of IL-2 and IL-15 signals in driving effector and memory CTL differentiation overlap.

However, IL-2 and IL-15 signals differ in magnitude, timing and context. While IL-2 binds its receptor as a soluble molecule, IL-15 is presented in trans by surface-bound IL-15Rα [3, 4], restricting the most potent IL-15 signals to periods of cell-cell contact, such as during the interaction of a T cell with an antigen presenting cell (APC). In support of this idea, dendritic cells are a key source of IL-15 for memory T cell homeostasis and survival [5]. Furthermore, expression of the high-affinity IL-2R is largely restricted to the first few days of the response, whereas IL-15 signals are presumably available to T cells during the initiation of the T cell response as well as during memory maintenance. It is possible, therefore, that these differences can be invoked to explain the distinct biological impacts of IL-2 and IL-15 on the T cell

response. In this scenario, IL-15, rather than sharing a role with IL-2 during the primary response, could have opposing functions, such as have been suggested in the respective roles of IL-2 and IL-15 in driving the differentiation of effector and memory T cells [6, 7].

Because IL-15 is highly related to IL-2 and shares a similar signaling apparatus, we hypothesized that IL-15 signals during the primary response could cooperate with IL-2 and compensate for the lack of IL-2 signals in the differentiation of $CD8^+$ effector and memory T cells.

Materials and methods

Mice and infections

6 to 8 week old C57BL/6 (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6NTac-IL15tm1Imx (IL-15-deficient) were purchased from Taconic Farms (Germantown, NY). WT and IL-2Rα-deficient P14 TCR transgenic mouse colonies were maintained at the University of Utah. All animal experiments were conducted with the approval of the IACUC committee at the University of Utah. LCMV Armstrong 53b was grown in BHK cells and titered in Vero cells [8]. Mice were infected intraperitoneally (i.p.) with 2×10^5 plaque-forming units (PFU). Recombinant *Listeria monocytogenes* expressing the LCMV GP33-41 peptide (Lm-gp33, generated using described methods) was propagated in BHI broth and agar plates as previously described [9-11]. Prior to infection, the bacteria were grown to log phase and concentration determined by measuring the O.D. at 600 nm (O.D. of 1 = 1×10^9 CFU/ml). For secondary challenges, mice were injected intravenously (i.v.) with 2×10^5 colony forming units (CFU).

Cell suspensions and adoptive transfers

Splenocytes and lymph node cells were harvested at the indicated time points and re-suspended in RPMI 1640 supplemented with 10% FBS and antibiotics. Liver and lung lymphocytes were harvested by collagenase digestion as previously described [12]. Untouched $CD8^+$ P14 T cells were isolated from the spleens and lymph nodes of WT or IL-2Rα-deficient P14 bone marrow chimeras by incubation with a biotinylated antibody cocktail followed by anti-biotin magnetic beads and depletion on a magnetic column, per manufacturer's recommendations (Miltenyi). In addition, we added biotinylated CD44 antibody (eBiosciences, San Diego, CA) to eliminate $CD44^{hi}$ "memory phenotype" P14. TCR transgenic T cell purity was assessed by staining with CD44, Vα2 and Vβ8.1 antibodies, followed by flow cytometric analysis. WT ($Thy1.1^+$) and IL-2Rα-deficient ($Thy1.1^+Thy1.2^+$) P14 were mixed 1:1 and co-injected i.v. into naïve B6 ($Thy1.2^+$) mice at the indicated doses one day prior to infection.

Peptide restimulation and intracellular cytokine staining

Splenocytes were resuspended in RPMI 1640 containing 10% fetal bovine serum and supplemented with antibiotics and L-glutamine. Mice were re-stimulated with 0.1 μM H-$2D^b$-restricted peptide (gp_{33-41}) in the presence of Brefeldin A (1 μg/ml GolgiPlug). Cells were stained with cell surface antibodies, permeabilized and stained with cytokine antibodies (specific to IFNγ, TNFα and IL-2) using a kit per manufacturer's instructions (BDBiosciences, Mountain View, CA).

Antibodies and flow cytometry

Fluorescent dye-conjugated antibodies were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA) or BDBiosciences (Mountain View, CA) with the following specificities: CD8, Thy1.1, Thy1.2, CD45.1, Va2, Vb8.1, CD44, IL-7Rα, KLRG1,

CD27, CD62L, CXCR3, CD43, CD122, TNFα, IL-2 and IFNγ. Cell surface antibody staining was done in PBS containing 2% FBS, and intracellular cytokine staining was performed as described above. For T-bet and Granzyme B staining, cells were permeabilized using the same buffers as for intracellular cytokine staining (BDBiosciences, Mountain View, CA). For Eomes, cells were permeabilized and stained using the same buffers as those used for the anti-FoxP3 antibody per the manufacturer's instructions (eBioscience, San Diego, CA). Multi-parameter (6-7 color) analysis of antibody-stained cells was performed on a FACSCanto II flow cytometer (BDBiosciences, Mountain View, CA) and results analyzed using FlowJo software (TreeStar). Cell sorting was with on a FACSVantage (BDBiosciences, Mountain View, CA) at the University of Utah FACS core facility.

Results

IL-2 and IL-15 jointly promote the emergence and survival of effector CTL

In order to probe a joint role for IL-2 and IL-15 signals to $CD8^+$ T cells, we adoptively co-transferred WT and IL-2Rα-deficient P14 cells into either WT or IL-15-deficient hosts. Because mouse $CD8^+$ T cells are not an in vivo source of IL-15 in mice, we were able to assess the response of P14 T cells in the absence of either IL-2 or IL-15 signals, or both. At the peak of the primary response (day 8 postinfection), the absence of either IL-2 or IL-15 alone resulted in a similar decrease (~2-3-fold) in the number of end-stage effector cells ($KLRG1^{hi}IL\text{-}7R^{lo}$). The combined absence of both IL-2 and IL-15 signals to $CD8^+$ T cells resulted in a further significant decrease in the number of effector CTL cells at the peak of the response, as compared to the absence of IL-2 or IL-15 signals alone (Fig. 3.1A, B). By day 42 postinfection, we observed a 10-20-fold decrease in the number of terminal effector phenotype cells in the absence of IL-2 signals, as compared

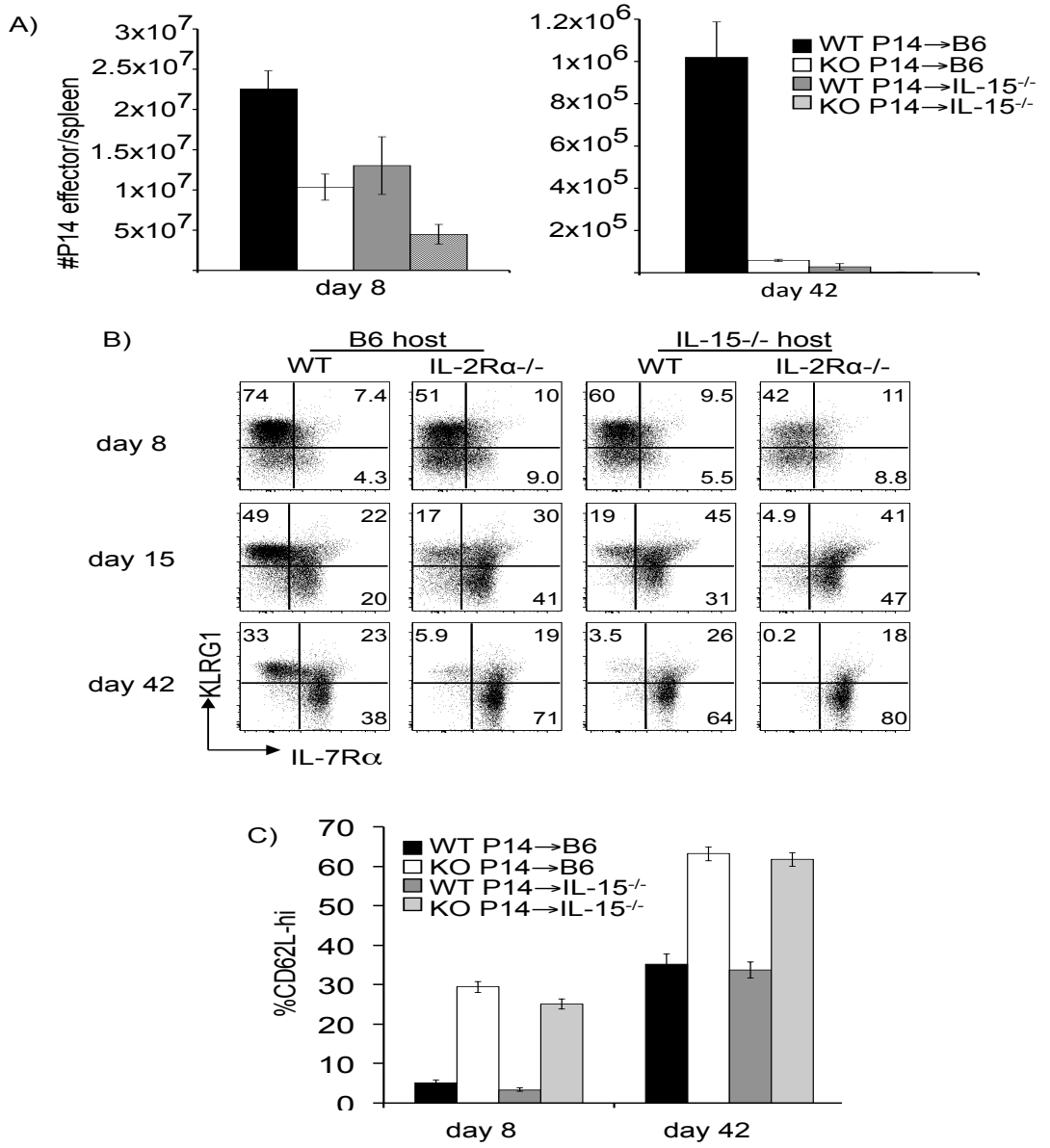

Figure 3.1 IL-2 and IL-15 jointly promote the persistence of terminal effector phenotype, but not long lived effector memory CD8+ T cells. We transferred 5x10^3 WT and IL-2Rα-/- P14 into B6 and IL-15-/- hosts and infected with LCMV. A) The bar graphs display the number of terminal effector phenotype (KLRG1-hi, IL-7Ra-lo) WT and IL-2Rα-/- P14 responders in B6 or IL-15-/- hosts at days 8 or 42 postinfection. Error bars indicate SEM (n=3). The decrease of P14 cells in the absence of both cytokines, as compared to each cytokine alone, is statistically significant at both time points (p<0.05). B) Representative flow plots indicate the relative frequencies of effector (KLRG1-hi, IL-7Ra-lo) and memory (KLRG1-lo, IL-7Ra-hi) phenotype cells among WT and IL-2Rα-/- P14 responders in B6 or IL-15-/- hosts at the indicated time points postinfection. C) The bar graph indicates the relative frequency of CD62L-hi cells among WT and IL-2Rα-/- memory phenotype P14 responders (KLRG1-lo, IL-7Ra-hi) in B6 or IL-15-/- hosts at the indicated time points postinfection. Error bars represent the SEM (n=3). Results are representative of 3 separate experiments.

to WT responses. The absence of IL-15 resulted in a similar decrease in the number of terminal effector phenotype cells at this time point. In the joint absence of IL-2 and IL-15 signals, this population was essentially undetectable, indicating a joint role for IL-2 and IL-15 in the differentiation and/or persistence of terminal effector phenotype $CD8^+$ T cells (Fig. 3.1A, B). These findings were independent of IL-15R expression, as WT and IL-2Rα-deficient P14 cells expressed similar levels of IL-15Rβ (CD122) regardless of the host (Fig. 3.2). Furthermore, differences could not be attributed to a lack of cell division, as similar frequencies of P14 cells expressed the cell cycle marker Ki-67 regardless of the presence of IL-15 (data not shown).

We further sought to determine whether IL-15 played a joint role with IL-2 in promoting the differentiation of effector memory $CD8^+$ T cells, as measured by CD62L expression. In this case, however, IL-15 appeared to play no role. While the absence of IL- 2 signals resulted in a more rapid skewing of the IL-$7R\alpha^{hi}$ memory precursor populations to a $CD62L^{hi}$ central memory-like phenotype, the absence of IL-15 signals had no such effect, either alone or in combination with the absence of IL-2 signals (Fig. 3.1C). Because IL- 15 is required for the homeostatic division and maintenance of $CD8^+$ memory T cells [13], we did not assess later memory time points, focusing instead on the role of IL-15 in the initial establishment of memory. These findings indicated that IL-15 might play a joint role with IL-2 in promoting the differentiation and/or survival of effector $CD8^+$ T cells but suggested distinct roles for these two cytokines in the differentiation of $CD8^+$ memory T cell subsets. While IL-15 promotes the survival and turnover of $CD8^+$ memory T cells over long periods of time, we saw little impact on $CD8^+$ memory T cell numbers in the absence of IL-15 at the early memory time points assessed in this study, consistent with prior reports [14].

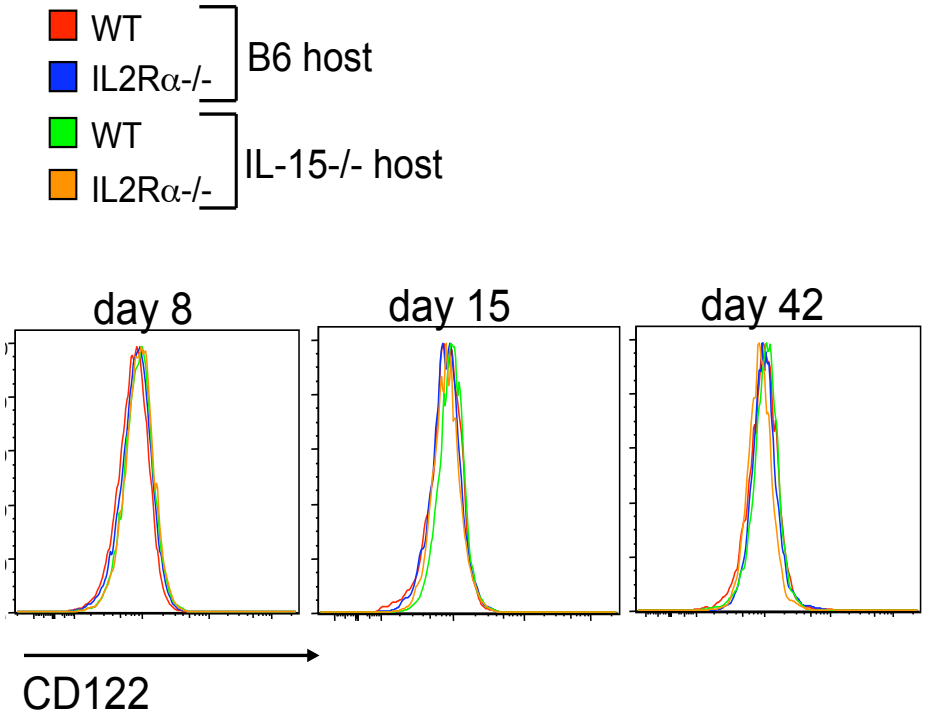

Figure 3.2 IL-2Rα-deficient and WT CD8+ memory T cells express similar levels of CD122 in WT and IL-15-/- hosts. WT and IL-2Rα-/- P14 (5000 of each) were co-transferred into B6 or IL-15-/- mice, followed by infection with LCMV one day later. Representative flow plots (n=3-4/time point in each group) indicate CD122 (IL-2Rβ) expression by P14 CTL of the indicated genetic background in the indicated hosts at day 8, 15 or 42 postinfection. Results are representative of three separate time courses.

Disparate roles for IL-2 and IL-15 in the differentiation and survival of KLRG1^{hi}IL- 7Rα^{lo} effector CTL

We considered two possible roles for IL-15 in promoting effector CTL populations. First, IL-15 signals during the priming and expansion phase might be important for their differentiation, similar to the role of IL-2. Our initial transfers of P14 cells into IL-15-deficient animals seemed to imply that this could be the case at least in part, as fewer terminal effector phenotype CTL were present at the peak of the primary response in the absence of IL-15 or IL-2 signals alone, and further decreased in their joint absence. On the other hand, IL-15 has been shown to play a key role in the survival of KLRG1^{hi}IL-7R^{lo} terminal effector phenotype CTL during the contraction phase [15-17], and we considered as an alternative that IL-15 was required only for the survival of these cells, not their differentiation.

In order to distinguish a potential differentiation role for IL-15 during the primary response from its known survival role thereafter, we limited, through adoptive transfer, the availability of IL-15 signals to WT or IL-2Rα-deficient P14 responders to the primary phase (days 0-8) or the contraction phase (days 8-42) of the T cell response. We co-transferred WT and IL-2Rα-deficient P14 in into B6 mice and infected with LCMV. At day 8 postinfection, WT and IL-2Rα-deficient P14 CTL were harvested from the spleen and transferred into infection-matched B6 or IL-15-deficient secondary hosts. We subsequently analyzed the persistence of WT or IL-2Rα-deficient terminal effector phenotype cells that lacked IL-15 signals during the primary response only, during the contraction phase only, or both. As before, we found that the absence of IL-15 during both the primary response and the contraction phase severely curtailed the persistence of KLRG1^{hi}IL-7Rα^{lo} effector cells and that the effect was exacerbated in the additional absence of IL-2 (Fig. 3.3). Similar results were observed when IL-15 signals were absent

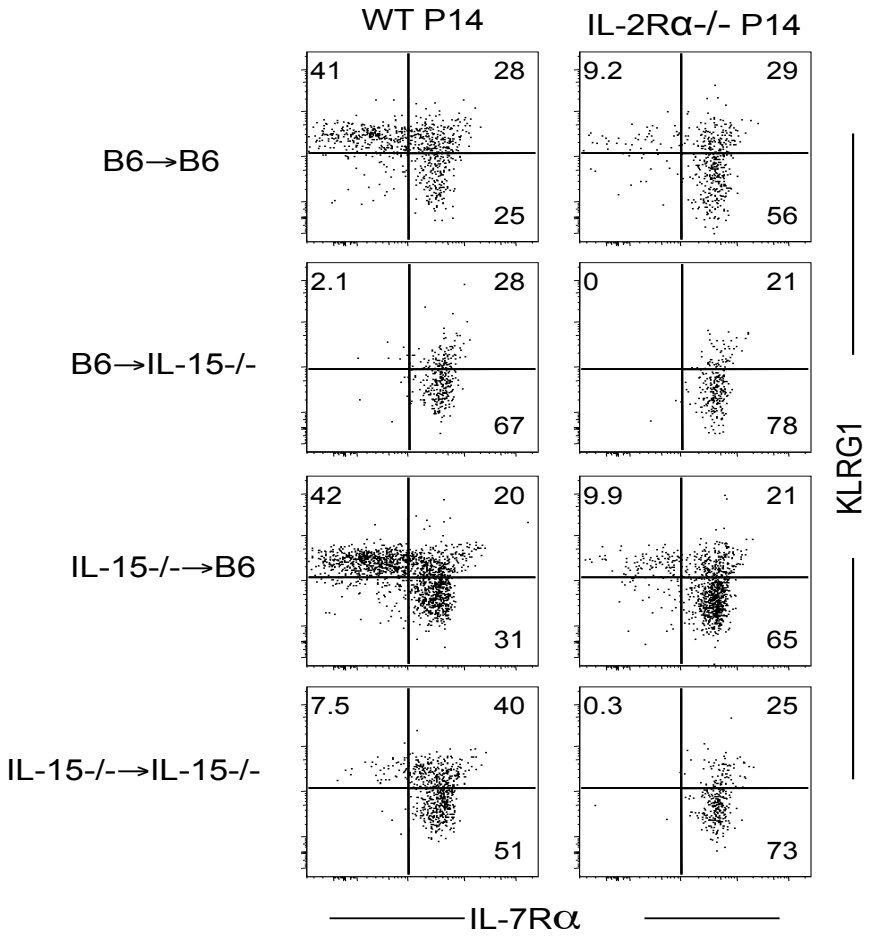

Figure 3.3 IL-15 is required for the survival but not differentiation of terminal effector phenotype CTL. We transferred 5x10^3 WT and IL-2Rα-/- P14 into B6 and IL-15-/- hosts and infected with LCMV. At day 8 postinfection, we isolated CD8+ T cells from their spleens using magnetic beads and transferred them to infection-matched secondary B6 or IL-15-/- hosts. Representative flow plots indicate the relative frequencies of effector (KLRG1-hi, IL-7Rα-lo) and memory (KLRG1-lo, IL-7Rα-hi) phenotype cells among WT and IL-2Rα-/- P14 responders in the spleens of secondary hosts at day 42 postinfection.

during the contraction phase only (Fig. 3.3) In contrast, the absence of IL-15 during the primary response alone resulted in levels of effector CTL levels similar to those seen following transfer of WT and IL-2Rα-deficient P14 into WT B6 hosts (Fig. 3.3). These findings conclusively demonstrate that in contrast to IL-2 the primary role for IL-15 in this setting is the survival of KLRG1^{hi} terminal effector phenotype CTL, not their differentiation.

IL-15 is not required for secondary CD8$^+$ T cell expansion and effector differentiation

We next assessed whether IL-15 shared an overlapping role with IL-2 in the differentiation of CD8$^+$ memory T cells capable of secondary responses. We co-transferred 500 WT and IL-2Rα-deficient P14 into B6 or IL-15-deficient mice and infected with LCMV as previously. At 42 days postinfection, mice were rechallenged with Lm-gp33. WT P14 memory cells expanded robustly by day 5 postrechallenge regardless of the presence or absence of IL-15 signals (Fig. 3.4A). They also differentiated into secondary effector CTL as determined by expression of IL-7Rα and KLRG1 (Fig. 3.4B) and their cytokine production profile (Fig. 3.4C). As observed previously, IL-2Rα-deficient memory cells responded poorly to secondary challenge and failed to acquire phenotypic or functional (cytokine-producing) characteristics indicative of secondary effector differentiation. However, this phenotype was not exacerbated in the absence of IL-15, again indicating that the functional role of IL-2 and IL-15 in CTL memory differentiation and survival were non-overlapping (Fig. 3.4A, B, C).

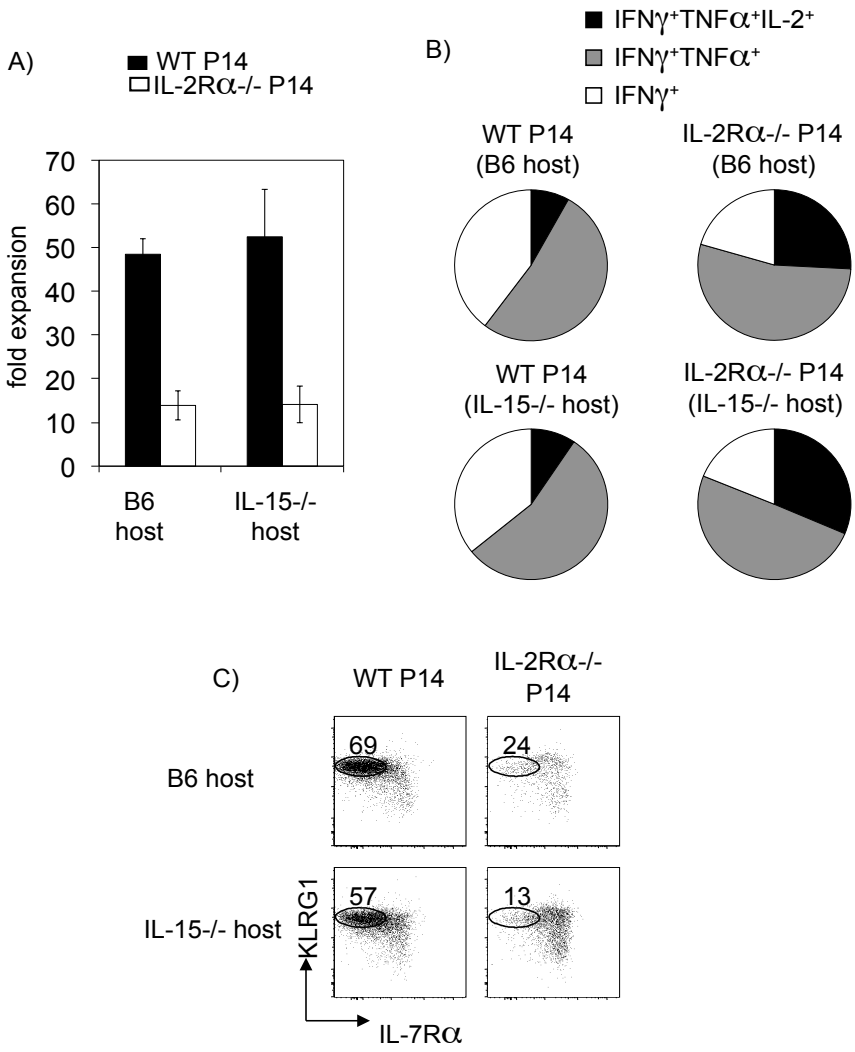

Figure 3.4 IL-15 is not required for the generation of secondary effector CTL. We transferred 500 WT and IL-2Rα-/- P14 into B6 and IL-15-/- hosts and infected with LCMV. At day 42 postinfection, we rechallenged mice with Lm-gp33 and assessed recall responses in the spleen 5 days later. A) The bar graph indicates the fold expansion 5 days after rechallenge of WT and IL-2Rα-/- P14 responders in B6 or IL-15-/- hosts. Error bars indicate SEM (n=3/group). B) The pie charts indicate the average frequency of WT or IL-2Rα-/- P14 secondary responders in WT or IL-15-/- hosts that make IFN-gamma after 4-hour restimulation and that also produce TNFα and/or IL-2 (n=3). C) Representative plots indicate the relative frequency of terminal effector phenotype secondary responders (KLRG1-hi, IL-7Rα-lo) among WT and IL-2Rα-/- P14 cells in WT or IL-15-/- hosts.

Discussion

In the absence of IL-2 signals, effector CTL undergo massive primary expansion. It is possible that the milieu of growth and inflammatory factors available during the infectious burst can compensate for the lack of IL-2 signals. This hypothesis is supported by the fact that IL-2 is one member of a family of cytokines linked by their receptor usage. Because the signaling apparatus used by IL-2 and IL-15 is largely shared, it is presently unclear what the differences are in the signals that account for their distinct biological effects. While we find a role for IL-15 in the survival of $CD8^+$ effector T cells after antigen clearance, it does not play a role in the differentiation of this population during primary activation, nor is it required for the differentiation of functionally competent $CD8^+$ memory T cells. Instead, our findings suggest that IL-15 plays a fundamentally distinct role from that of IL- 2, promoting not the differentiation, but survival of $CD8^+$ memory T cells.

We find little role for IL-15 signals during the primary response, either alone or in combination with IL-2, in promoting effector or effector memory differentiation or programming the recall capacity of $CD8^+$ central memory T cells. Instead, the dominant role of IL-15 was to promote the survival of effector and memory populations after pathogen clearance. While excess IL-15 signals may serve as an adjuvant or have immunotherapeutic benefit for $CD8^+$ T cell responses, our findings suggest that in settings of acute infection, physiological IL-15 signals to T cells during the primary response do not play a significant role in $CD8^+$ effector and memory T cell differentiation, particularly as compared to IL-2.

It is not clear, however, whether the programming of functional memory cells by IL-2 represents a unique signal from IL-2 that other γ_c-family member cytokines are unable to deliver, or if it represents a common signal, that any member of the family

could redundantly deliver during T cell activation and differentiation, were it present in sufficient amounts and its receptor expressed.

Our results suggest that IL-15 signals do not overlap with IL-2 signals, but this may reflect differences in receptor expression and cytokine availability resulting in different contexts of cytokine stimulation. Because IL-15 is expressed in trans [4], T cells must be in close proximity to potential IL-15 producers, whereas, IL-2 is secreted into the environment. It is unclear the extent to which IL-15 signals are available to differentiating CTLs during the primary response, but IL-15 presented by dendritic cells has been shown to induce homeostatic proliferation of memory T cells [5]. It is possible that the levels of IL-15 available to $CD8^+$ T cells differ depending on the nature of the pathogenic stimulus, and our results do not rule out a potential role for IL-15 during primary $CD8^+$ T cell differentiation if present at high enough concentrations in other infectious model systems.

References

1. Alves, N.L., F.A. Arosa, and R.A. van Lier, *Common gamma chain cytokines: dissidence in the details.* Immunology letters, 2007. **108**(2): p. 113-20.

2. Bodnar, A., et al., *A biophysical approach to IL-2 and IL-15 receptor function: localization, conformation and interactions.* Immunology letters, 2008. **116**(2): p. 117-25.

3. Burkett, P.R., et al., *Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis.* The Journal of experimental medicine, 2004. **200**(7): p. 825-34.

4. Sandau, M.M., et al., *Cutting edge: transpresentation of IL-15 by bone marrow-derived cells necessitates expression of IL-15 and IL-15R alpha by the same cells.* Journal of immunology, 2004. **173**(11): p. 6537-41.

5. Stonier, S.W., et al., *Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation.* Blood, 2008. **112**(12): p. 4546-54.

6. Manjunath, N., et al., *Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes.* The Journal of clinical investigation, 2001. **108**(6): p. 871-8.

7. Weninger, W., et al., *Migratory properties of naive, effector, and memory CD8(+) T cells.* The Journal of experimental medicine, 2001. **194**(7): p. 953-66.

8. Ahmed, R., et al., *Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence.* The Journal of experimental medicine, 1984. **160**(2): p. 521-40.

9. Shen, H., et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(9): p. 3987-91.

10. Slifka, M.K., et al., *Antiviral cytotoxic T-cell memory by vaccination with recombinant Listeria monocytogenes.* Journal of virology, 1996. **70**(5): p. 2902-10.

11. Williams, M.A., E.V. Ravkov, and M.J. Bevan, *Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory.* Immunity, 2008. **28**(4): p. 533-45.

12. Williams, M.A. and M.J. Bevan, *Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells.* Journal of immunology, 2004. **173**(11): p. 6694-702.

13. Surh, C.D. and J. Sprent, *Homeostasis of naive and memory T cells.* Immunity, 2008. **29**(6): p. 848-62.

14. Wherry, E.J., et al., *Homeostatic proliferation but not the generation of virus specific memory CD8 T cells is impaired in the absence of IL-15 or IL-15Ralpha.* Advances in experimental medicine and biology, 2002. **512**: p. 165-75.

15. Joshi, N.S., et al., *Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor.* Immunity, 2007. **27**(2): p. 281-95.

16. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin.* Nature immunology, 2005. **6**(12): p. 1236-44.

17. Rubinstein, M.P., et al., *IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response.* Blood, 2008. **112**(9): p. 3704-12.

CHAPTER 4

DIFFERENT REQUIREMENTS FOR STAT5 IN PRIMARY AND SECONDARY $CD8^+$ T CELL RESPONSES

Introduction

Environmental signals, including cytokine signals, received by $CD8^+$ T cells during the primary immune response have an essential role in effector and memory $CD8^+$ T cell differentiation [1]. The common gamma chain (γ_c) family cytokine IL-2 has been shown to drive $CD8^+$ T cell effector and memory differentiation [2-5]. We and others have shown a role for IL-2 during the primary immune response in driving differentiation of effector CTL [2-4], as well programming of memory $CD8^+$ T cells capable of robust recall responses [3, 5].

How $CD8^+$ T cells integrate signals by cytokines to determine fate decisions are not fully understood. Specifically, how do cytokines, such as IL-2, induce signaling pathways that result in long-lived memory $CD8^+$ cells capable of protective recall responses? Several transcription factors, including T-bet [6-11], Eomes [6, 8, 12, 13], Blimp-1 [14-18], and Bcl-6 [14, 19, 20], have been shown to have crucial roles in $CD8^+$ T cell effector differentiation and effector versus memory fate decisions. In the case of T-bet [6, 8-11] and Blimp-1 [14, 16-18], increased activity promotes a more differentiated terminal effector state in $CD8^+$ T cells. On the other hand, Eomes [6, 8, 12, 13] and Bcl-6 [14, 19, 20] are associated with establishment of long-lived memory $CD8^+$ T cell populations. In the case of effector $CD4^+$ T cell differentiation, connections between cytokines and transcriptional programs is relatively well described and in several lineages involves STAT proteins [21]. In contrast, the molecular connection between environmental signals, such as cytokines, received by $CD8^+$ T cells during an immune response and the resulting transcriptional outcome driving differentiation towards terminal effector versus memory $CD8^+$ T cell fate is less clear.

We have shown a crucial role for the cytokine IL-2 in the differentiation of effector and memory $CD8^+$ T cells [3, 5]. Signals from IL-2 during the primary response to infection are critical in promoting the differentiation of effector and effector memory

CTL [3]. Moreover, IL-2 signals received by CTL during the primary response program memory CD8+ T cells capable of robust secondary responses [3, 5] and secondary effector differentiation [3]. We sought to determine the signaling pathways induced by IL-2 during a primary immune response that are involved in $CD8^+$ T cell fate decisions and memory $CD8^+$ T cell programming. The JAK3/STAT5, PI3K/Akt, and MAPK signaling pahways are potently activated by IL-2 [22]. Of these pathways, PI3K/Akt and MAPK are activated by a variety of other cytokine receptors, TCR ligation, and costimulatory signals [23-26] during T cell activation. Other γ_c cytokines in addition to IL-2, such as IL-7 and IL-15, also activate JAK3/STAT5, but the timing and context of this signaling differ due to cytokine availability and differential regulation of the specific receptor subunits [27]. IL-2 potently activates STAT5 during the differentiation of effector CTL. While IL-15 and IL-7 are present during the effector CTL response, their primary role appears to be in the maintenance of memory T cells after antigen clearance [27, 28]. Moreover, IL-15 and IL-7 signals are not required for the programming of memory $CD8^+$ T cells [3, 29, 30] as is the case with IL-2 [3, 5]. Therefore, IL-2 is the most likely catalyst for robust STAT5 activation during primary CTL responses, and we hypothesized that IL-2-mediated activation of STAT5 was a key step in the differentiation of highly functional $CD8^+$ memory T cells.

Two isoforms of STAT5 exist, STAT5a and STAT5b, which have redundant functions in T cell homeostasis [31, 32]. Mice with deletion of both *stat5a/b* fail to develop T cells [33, 34], demonstrating that STAT5 is essential for T cell development. Recent reports examined the role of STAT5 in the survival of effector and memory $CD8^+$ T cells after pathogen clearance [35, 36]. In the absence of STAT5, effector $CD8^+$ T cells show reduced accumulation at the peak of the primary response to infection, possibly due to their inability to induce Bcl-2 via STAT5 in response to IL-7 and IL-15 [36]. Forcing expression of a constitutively active form of STAT5 increased the numbers of

effector CTL at the peak of the primary response to LCMV, and augmented survival of all CD8+ T cell subsets through contraction and memory [35]. This was possibly due to increased augmentation of as well as access to PI3K/Akt signaling in the presence of STAT5 signaling. These studies support STAT5 signaling in effector and memory $CD8^+$ T cell survival, mainly in the context of IL-15 and IL-7 signaling. However, the function of STAT5 signals during the primary response to infection, as driven by IL-2, in CTL differentiation, fate decisions, and memory $CD8^+$ T cell programming has not been examined.

To determine the contribution of STAT5 to $CD8^+$ effector and memory T cell differentiation, we employed a model in which *stat5a/stat5b* are inducibly deleted upon CTL activation. We find that STAT5 is broadly important for all effector $CD8^+$ T cell subsets during the primary response to acute infection, but is selectively important for the survival of terminal effector phenotype CTL and tissue residing memory $CD8^+$ T cells during contraction. After contraction, memory $CD8^+$ T cell populations formed in the absence of STAT5 are readily detectable at 6 weeks postinfection. While STAT5 was required for robust expansion and survival of primary effector CTL, STAT5-deficient memory $CD8^+$ T cells mounted robust recall responses comparable to wild-type levels and underwent effective secondary effector CTL differentiation. Our findings highlight a differential requirement for survival signals mediated by STAT5 during primary and secondary $CD8^+$ T cell responses. Moreover, our data suggest that IL-2 driven differentiation and programming of memory $CD8^+$ T cells with robust recall potential is largely STAT5 independent.

Materials and methods

Mice and infections

4 to 6 week old B6.PL-*Thy1*^{a}/CyJ (B6.PL, Thy1.1^{+}) and C57BL/6 (B6, Thy1.2^{+}) mice were purchased from Jackson Laboratories (Bar Harbor, ME). GzB-Cre [37], Rosa26-^{fl}/stop/^{fl}-YFP (RosaYFP) [38] (Jackson Laboratories), and stat5$^{fl/fl}$ [39] mouse colonies were maintained at the University of Utah (Salt Lake City, UT). All experiments were performed with the approval of the IACUC committee at the University of Utah. Lymphocytic choriomeningitis virus (LCMV) strains Armstrong 53b and clone 13 were grown in BHK cells and titered in Vero cells as described [40]. Primary infection was at a dose of 2 x 10^5 PFU i.p. For secondary challenges, mice were given 2 x 10^6 PFU LCMV-Cl.13 i.v. or 2 x 10^5 PFU LCMV-Arm i.p. Recombinant *Listeria monocytogenes* expressing the LCMV GP33-41 peptide (Lm-gp33, generated using described methods) was propagated in BHI broth and agar plates as previously described [41-43]. Prior to infection, the bacteria were grown to log phase and concentration determined by measuring the O.D. at 600 nm (O.D. of 1 = 1 x 109 CFU/ml). For secondary challenges, mice were injected intravenously (i.v.) with 10,000 colony forming units (CFU).

Irradiation chimeras

Host B6.PL (Thy1.1^{+}) mice were given a dose of 900 rads using the analytical x-ray irradiator in the mouse vivarium at the University of Utah. The next day, mice recieved 5 x 10^6 bone marrow (BM) cells harvested from the femurs and tibias of donor mice as indicated. BM cells were prepared by RBC lysis and depletion of CD3^{+} cells using biotinylated anti-CD3 antibody (eBioscience, San Diego, CA), anti-biotin magnetic beads (Miltenyi Biotec, Auburn, CA), and passage through magnetic column following manufacturer's guidelines (Miltenyi Biotec). WT B6.PL (Thy1.1^{+}) BM was mixed 1:1 with

either GzB-Cre-RosaYFP-stat5$^{wt/wt}$(Thy1.2^{+}) or GzB-Cre-RosaYFP-stat5$^{fl/fl}$(Thy1.2^{+}) BM and injected i.v. into the irradiated hosts. After 8-10 weeks, reconstitution within the CD8^{+} T cell population was determined using antibodies to the congenic markers Thy1.1 and Thy1.2.

Cell suspensions and cell sorting

Splenocytes and lymph node cells were harvested at indicated time points postinfection and placed in single cell suspension in RPMI 1640 with 10% FBS, L-glutamine, and Pen/Strep prior to further analysis. Liver lymphocytes were harvested and subjected to collagenase digestion as previously described [44]. Cell sorting of CD8^{+}YFP^{+} cells was performed using a FACS Aria II cell sorter (BD Biosciences) at the University of Utah FACS Core Facility, followed by immediate adoptive transfer by i.v. injection into the indicated secondary hosts.

Flow cytometry and analysis

Antibodies (Abs) conjugated to fluorescent dyes were purchased from eBioscience (San Diego, CA), Biolegend (San Diego, CA), or BD Biosciences (Mountain View, CA). Abs were specific for the following antigens: CD8, Thy1.1, Thy1.2, KLRG1, IL7R α , CD62L, T-bet, Eomesodermin (Eomes), Bcl6, GranzymeB, STAT5-pY694. For cell surface staining, single cell suspensions were incubated with Abs in FACS Buffer (PBS with 2% FBS and 0.02% sodium azide). For intracellular staining of T-bet and GranzymeB, cells were permeabilized and stained using the BD Cytofix/Cytoperm™ kit and manufacturer's instructions (BD Biosciences). For intracellular staining of Eomes, cells were pre-fixed in 0.5% PFA to preserve the YFP signal, washed twice, then fixed using FoxP3 Fix/Perm Buffer kit and manufacturer's instructions (eBioscience). To detect STAT5-pY694, the BD Phosflow™ kit and manufacturer's protocol were followed (BD Biosciences). H-2D^{b}/GP$_{33-41}$ and H-2D^{b}/NP$_{396-404}$ were prepared as previously

described [45, 46]. Multiparameter analysis of stained cells was performed using a FACSCanto II flow cytometer (BD Biosciences) and results were analyzed using FlowJo software (TreeStar, Ashland, OR).

Intracellular cytokine staining

Single cell suspensions in RPMI 1640 containing 10% FBS, L-glutamine, and penicillin/streptomycin were incubated with 0.1 uM GP_{33-41} or $NP_{396-404}$ peptides in the presence of GolgiPlug (BD Biosciences) for 5 hours per the manufacturer's instructions. Cells were stained with cell surface Abs, fixed and permeablized using a kit (BD Biosciences) and stained with fluorescently labeled anti-cytokine Abs specific to IFN-γ, TNF-α, and IL-2 (eBioscience).

CTL assays

We used a CTL assay as previously described [6]. EL-4 targets cells were incubated with 0.1uM GP_{33-41} peptide for 2 hrs at 37° C. Cells were washed and then incubated with sorted STAT5 WT or STAT5 CKO P14 CTLs for 2 hrs at CTL:target ratios ranging from 3:1 to 0.1:1. The percentage of Annexin V+ target cells was determined by FACS using Annexin V-APC apoptosis detection kit (eBioscience). Specific lysis was determined by comparison of killing of target cells without peptide loading.

Real-time PCR

RNA was extracted from sorted YFP^+CD8^+ T cells using Trizol (Invitrogen, Carlsbad, CA). cDNA was prepared from the RNA and qRT-PCR was performed using Superscript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. Primer sets used are as follows: Eomes: f-CCGCCCACTACAATGTTTTC, r-GAAATCTCCTGCCTCATCCA; T-bet: f-CCCACAAGCCATTACAGGAT, r-CCCTTGTTGTTGGTGAGCTT; Blimp-1: f-

CGGGATGAACATCTACTTCTACACT, r-TTTCTTTCACGCTGTACTCTCTCTT; Bcl-6: f-CCGGCTCAATAATCTCGTGAA, r-GGTGCATGTAGAGTGGTGAGTGA; FasL: f-CATCACAACCACTCCCACTG, r-TACTGGGGTTGGCTATTTGC; Prf1: f-GCAGCTGAGAAGACCTATCAGGAC, r-TCTGAGCGCCTTTTTGAAGTC; Bcl-2: f-GTGGTGGAGGAACTCTTCAGGGATG, r-GGTCTTCAGAGACAGCCAGGAGAAATC; Bim: f-CGGATCGGAGACGAGTTCA, r-TTCAGCCTCGCGGTAATCA; mcl-1: f-AGAGCGCTGGAGACCCTG, r-CTATCTTATTAGATATGCCAGACC; Bcl-XL: f-GTAGTGAATGAACTCTTTCGGGATGG, r-ACCAGCCACAGTCATGCCCGTCAGG. Real-time PCR and analysis was performed using a Roche LightCycler® 480 (Roche, Indianapolis, IN).

Results

STAT5 is required for accumulation of effector CTL during acute infection

In order to study the role of STAT5 signals in $CD8^+$ T cell effector and memory differentiation, we created a mouse bone marrow chimera model system in which *stat5a* and *stat5b* are deleted in activated $CD8^+$ T cells, and the resulting STAT5-deficient $CD8^+$ T cells are traceable by permanent expression of YFP (Fig. 4.1A). Bone marrow was harvested from mice with the following genetic components: Cre recombinase driven by the GranzymeB promoter [37] (GzB-Cre), RosaYFP reporter construct [38], and the *stat5a/b* genes flanked by loxP sites [39] ($stat5^{fl/fl}$), which we will subsequently refer to as "STAT5 conditional knockout (CKO)" bone marrow. The donor bone marrow was mixed 1:1 with wild-type B6.PL bone marrow ("WT") and transplanted into irradiated B6.PL hosts. This set of chimeras will subsequently be referred to as STAT5 CKO chimeras. A second set of bone marrow chimeras was made in an identical fashion, except the donor bone marrow contained the *stat5a/b* genes without LoxP sites

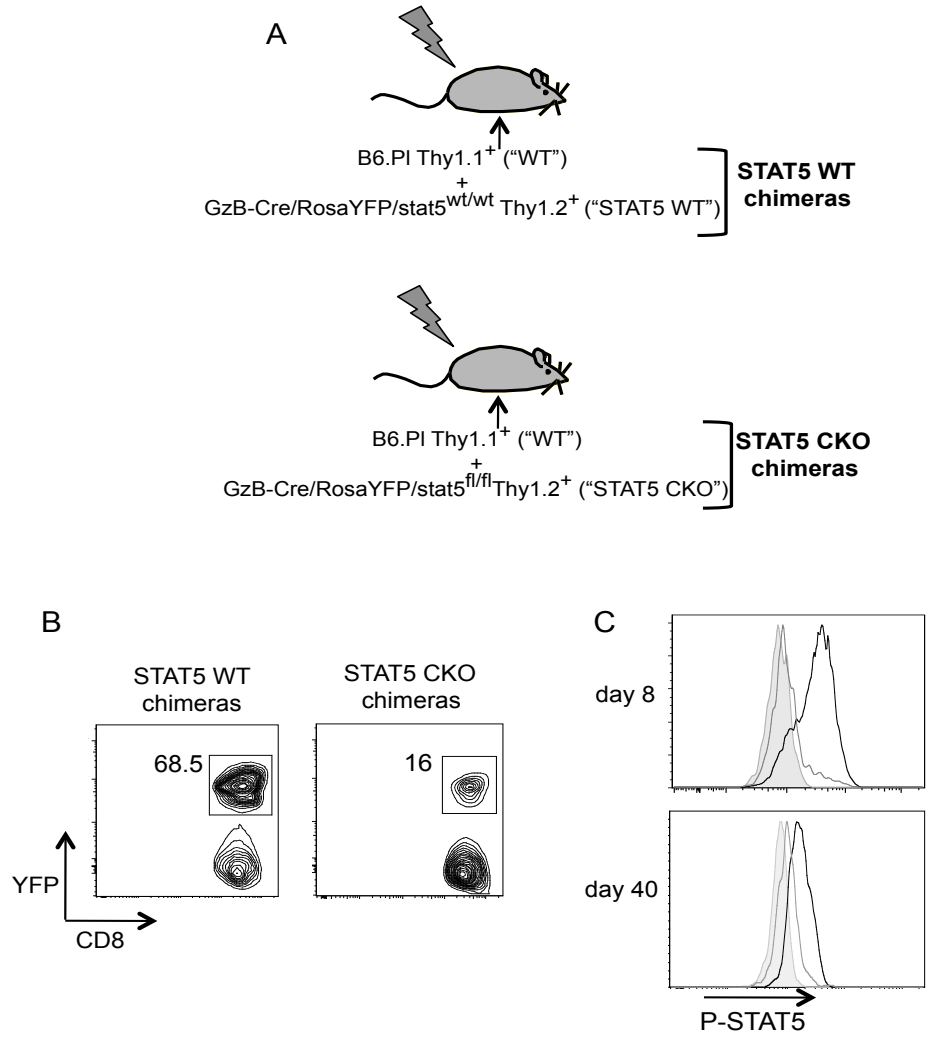

Figure 4.1 Validation of STAT5 CKO model system. *A,* Schematic showing creation of STAT5 WT and STAT5 CKO bone marrow chimeras for model system to study CD8+ T cell responses in the absence of STAT5. *B,* Representative flow plots showing CD8+YFP+ populations in the STAT5 (Thy1.2+) component of both STAT5 WT and STAT5 CKO chimeras at day 8 postinfection with LCMV. *C,* Splenocytes from STAT5 WT or STAT5 CKO Chimeras harvested on day 8 or day 40 postinfection were cultured with 500 U/mL mIL-2 then stained for phospho-STAT5. Representative histogram shows P-STAT5 levels in unstimulated controls (gray shaded), CD8+YFP+ T cells from STAT5 WT chimeras (black line), and CD8+YFP+ T cells from STAT5 CKO chimeras (dark gray line).

(stat5$^{wt/wt}$) and will be referred to as STAT5 WT chimeras (Fig 4.1A). We distinguished between STAT5 (Thy1.2$^+$) and B6.PL (Thy1.1$^+$) donor-derived T cells over the course of infection based on differential expression of congenic Thy1 alleles. In this model system, activation of Cre activity upon CTL activation, driven by Granzyme B induction, results in the deletion of LoxP-flanked stat5a/b as well as a LoxP-flanked stop codon preceding YFP under the control of the Rosa26 promoter. Because Granzyme B expression is one of the earliest events following CTL activation, permanent YFP expression serves as an effective surrogate marker for Cre-mediated gene deletion during CTL differentiation and expansion.

Following infection with LCMV-Armstrong, CD8$^+$ T cell expansion was evident in both STAT5 WT and STAT5 CKO chimeras, and both groups of chimeras cleared the infection as expected (data not shown). A clearly distinguishable CD8$^+$YFP$^+$ population was seen in both STAT5 WT and STAT5 CKO chimeras on day 8 postinfection (Fig. 4.1B). Importantly, we were able to confirm that STAT5 was absent from the CD8$^+$YFP$^+$ population within the STAT5 CKO chimeras at effector and memory time points (Fig. 4.1C).

Following LCMV infection, we observed a defect in the overall expansion of CD8$^+$YFP$^+$ effector CTLs in the spleen in the absence of STAT5. While STAT5 WT chimeras displayed similar expansion of CD8$^+$ T cells derived from either donor, STAT5 CKO chimeras displayed deficient expansion by CD8$^+$ T cells derived from the STAT5 CKO donor (Fig. 4.2A). By day 8 postinfection, the CD8$^+$ T cell population in the spleens of STAT5 CKO chimeras was almost entirely derived from the WT donor (Fig. 4.2B). This shows a decreased accumulation of STAT5 CKO CD8$^+$ T cells at day 8 postinfection compared to WT.

We examined STAT5 WT and STAT5 CKO CD8$^+$ T cells for differences in anti-apoptotic, pro-survival, or pro-apoptotic gene expression on day 8 postinfection with

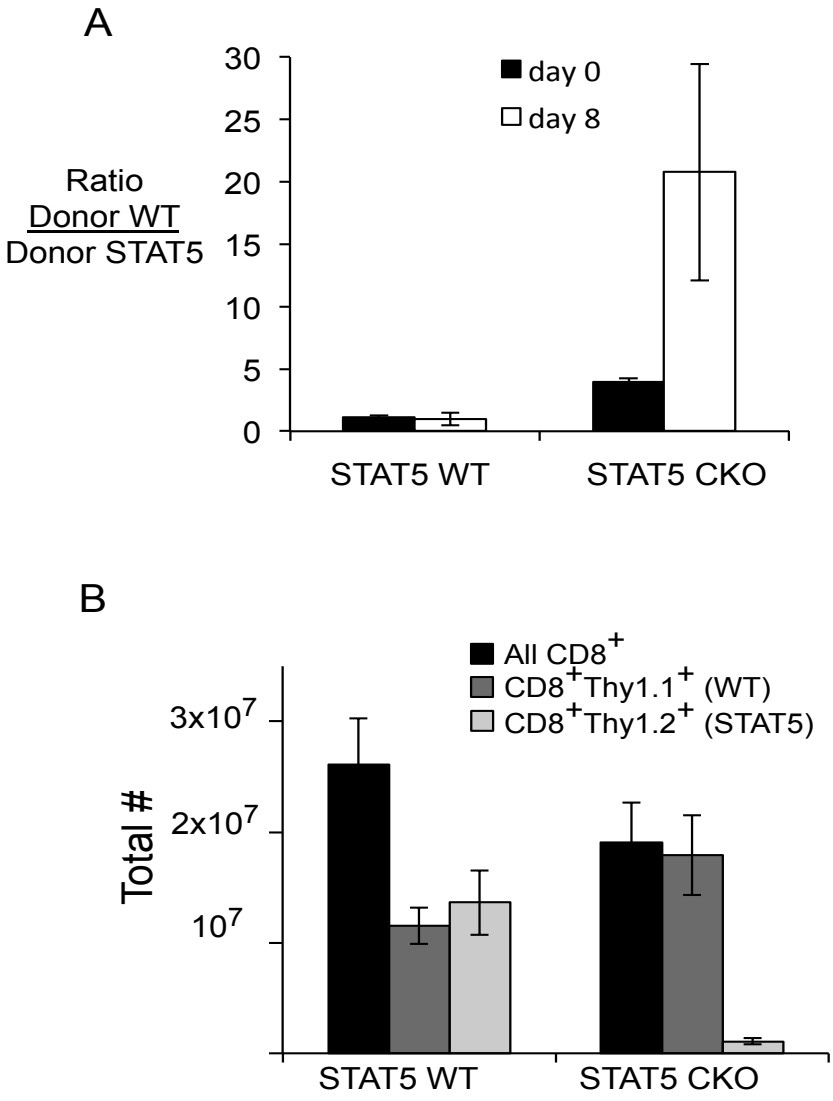

Figure 4.2 STAT5 is required for full primary expansion of CD8+ T cells. *A,* The ratio of "WT" to "STAT5" components in the CD8+ T cell compartment are shown for both sets of chimeras prior to (day 0) and day 8 post LCMV infection. *B,* Total numbers of CD8+ T cells from "WT" and "STAT5" components on day 8 post LCMV infection in STAT5 WT and CKO chimeras. Error bars indicate the SEM (n=3-4 mice per time point). Results are representative of 4-5 independent experiments.

Table 4.1 Gene expression in CD8+YFP+ WT STAT5 compared to CD8+YFP+ STAT5 CKO T cells day 8 postinfection with LCMV. Fold differences are significant for $p<0.05$, n=4 per group.

Gene	Fold expression in WT STAT5 compared to STAT5 CKO
Tbet	1.57 *(p=0.008)*
Blimp-1	1.82 *(p=0.021)*
Perforin	1.55 *(p=0.010)*
Bcl-6	0.23 *(p=0.041)*
EOMES	1.42 *(p=0.150)*
FasL	0.95 *(p=0.859)*
Bcl2	0.71 *(p=0.365)*
Bcl-X_L	1.77 *(p=0.046)*
Bim	1.17 *(p=0.642)*
Mcl-1	0.88 *(p=0.654)*

LCMV and found no significant differences in expression of the pro-survival genes Bcl-2 and Mcl-1 and no difference in expression of the pro-apoptotic gene Bim (Table 4.1). STAT5 WT CD8$^+$ T cells showed a modest increase in expression of Bcl-xL over STAT5 CKO CD8$^+$ T cells (Table 4.1).

We next examined how STAT5-deficiency affected antigen-specific CD8$^+$ T cells responding to acute infection by staining with MHC Class I tetramers for two immunodominant LCMV epitopes. While the frequency of H-2D^b/GP$_{33-41}$ and H-2D^b/NP$_{396-404}$ tetramer$^+$ cells was similar in the STAT5 WT and STAT5 CKO CD8$^+$ T cell populations (Fig. 4.3A), due to the overall loss in CD8$^+$ T cells the total numbers of STAT5 CKO antigen specific CD8$^+$ T cells was substantially lower than WT at the peak of the response in the spleen (Fig. 4.3B). Because measuring total numbers does not take into account the change in frequency of donor CD8$^+$ T cells seen between day 0 and day 8 postinfection (Fig. 4.2A), we normalized the CTL response in each chimera to the predicted response based on the ratio of donor WT to donor STAT5 WT or STAT5 CKO CD8$^+$ T cells prior to infection. We found that the STAT5 CKO antigen specific CD8$^+$ T cell response was approximately 4-fold lower than the wild-type response seen by STAT5 WT CTLs (Fig. 4.3C). Importantly, these differences were not due to inefficient deletion of STAT5 as antigen specific YFP$^+$ STAT5 CKO CTLs displayed virtually no STAT5 expression at day 8 postinfection (Fig. 4.3D). We conclude that STAT5 is an important survival signal for CD8$^+$ T cells undergoing expansion during primary immune challenge.

STAT5 influences CTL function but does not drive
CTL differentiation

To explore whether STAT5 influenced effector CTL differentiation, we examined the localization, expression of effector molecules and function of effector CTLs in the absence of STAT5. Antigen-specific wild-type and STAT5 CKO CD8$^+$ T cells were present

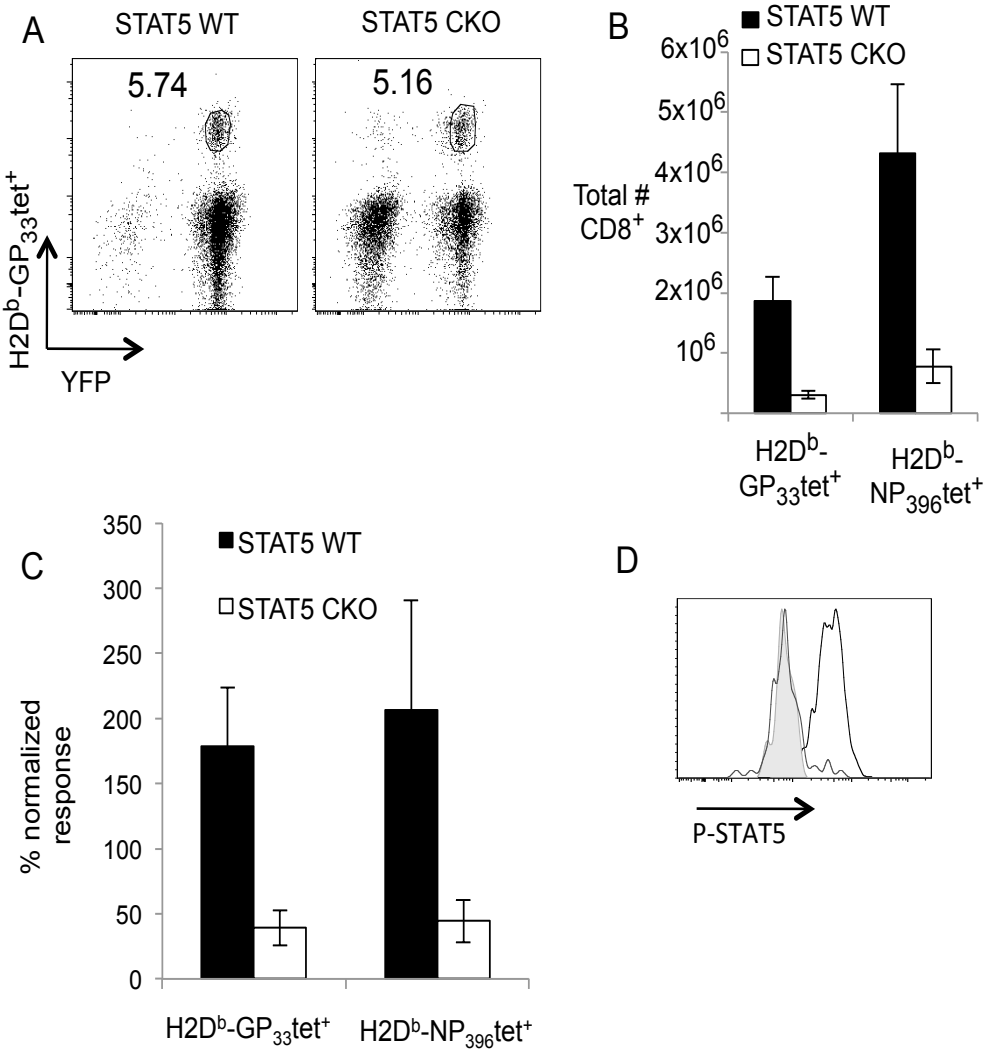

Figure 4.3 Antigen specific STAT5 CKO primary responses are reduced in number compared to wildtype. *A,* Representative flow plots show the frequency of antigen specific (H2Db-GP33-41 tetramer$^+$) YFP+ within the CD8+ population in the spleen on day 8 post LCMV infection. *B,* Total numbers of WT and STAT5 CKO CD8+ T cells on day 8 postinfection for two LCMV-specific epitopes. *C,* Differences in absolute numbers seen on day 8 postinfection for antigen-specific CD8+ T cells were normalized to reflect changing ratios of WT and STAT5 counterparts seen between day 0 and day 8 postinfection (Fig 1D). Graphs showing the % of normalized response are displayed for two antigen-specific CD8+ T cell populations in a representative experiment. Error bars represent the SEM (n=3-4 per group). *D,* Confirmation of deletion of STAT5 within antigen specific YFP+CD8+ T cells from STAT5 CKO Chimeras. Splenocytes from STAT5 WT and CKO Chimeras were harvest on day 8 post LCMV infection, surface stained for CD8 and tetramers, cultured with 500U/mL IL-2, then stained for P-STAT5. Representative histogram shows P-STAT5 levels in unstimulated controls (gray shaded) and tetramer$^+$YFP+CD8+ T cells from either STAT5 WT (black line) or STAT5 CKO chimeras (gray line).

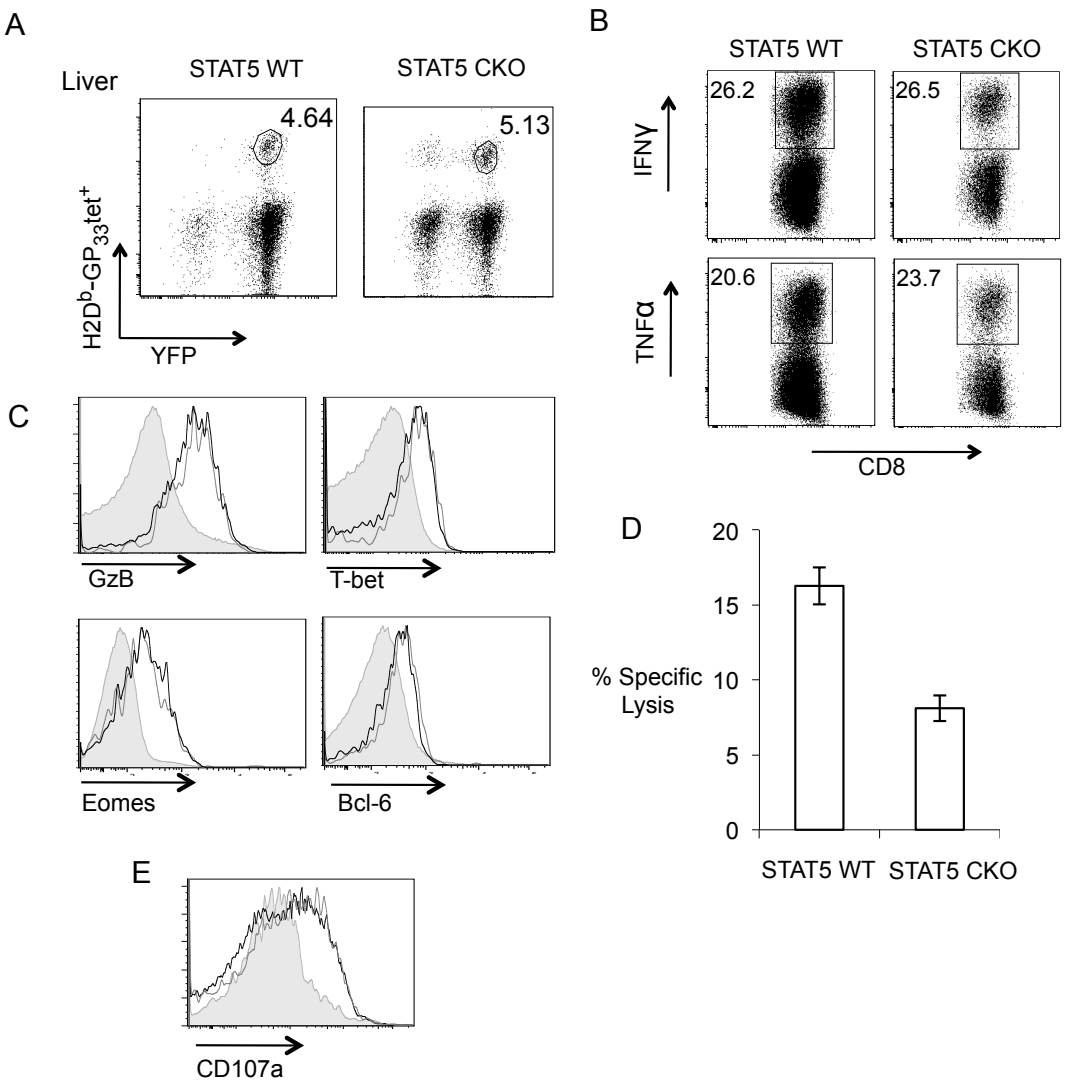

Figure 4.4 Effector CTL function is modestly reduced in the absence of STAT5. *A,* Representative flow plots show the frequency of antigen specific (H2Db-GP33 tetramer+) YFP+ within the CD8+ population in the liver on day 8 postinfection with LCMV. *B,* Cytokine expression was determined by ex vivo GP33-41 peptide restimulation of splenocytes on day 8 postinfection. Representative flow plots display IFN-γ and TNF-α production gated on CD8+YFP+ T cells from from STAT5 WT or STAT5 CKO chimeras. *C,* Intracellular expression of GranzymeB, T-bet, EOMES, and Bcl-6 on day 8 postinfection in STAT5 WT (black line) and STAT5 CKO(gray line) GP33 tetramer+ CD8+ T cells. Gray filled histograms display isotype control staining. *D,* Target cells loaded with GP33-41 peptide were incubated with STAT5 WT or STAT5 CKO CTL FACS sorted from spleens at day 8 postinfection. CTL induced lysis was measured by staining with Annexin V after 2 hrs. % specific lysis was determined by comparison to Annexin V^+ target cells that were not loaded with peptide. *E*, Degranulation measured by CD107a staining of ex vivo GP33 peptide stimulated splenocytes on day 8 postinfection. Error bars represent the SEM (n=3-4 per group). Results are representative of 3-4 independent experiments.

at similar frequencies in the liver of LCMV infected mice on day 8 postinfection (Fig. 4.4A), demonstrating that CTL form and traffic to peripheral sites of infection even in the absence of STAT5. Additionally, STAT5 CKO $CD8^+$ T cells produced IFN-γ and TNF-α at similar frequencies and levels upon peptide restimulation of splenocytes (Fig 4.4B) and liver lymphocytes (data not shown). STAT5 CKO tetramer binding $CD8^+$ T cells readily expressed similar levels of the cytolytic molecule GranzymeB (GzB) as STAT5 WT tetramer binding cells on day 8 postinfection (Fig. 4.4C). In addition, STAT5 CKO CTL degranulated normally upon peptide restimlulation, as measured by the surface expression of CD107a following peptide restimulation (Fig. 4.4E).

Modest differences in mRNA levels (Table 4.1) were seen for two transcription factors, T-bet and Eomes, that are associated with effector CTL [9, 11] and effector to memory $CD8^+$ T cell transition [8, 12], respectively. However, protein expression levels of T-bet and Eomes were not changed in the absence of STAT5 (Fig. 4.4C). Another transcriptional access affecting $CD8^+$ T cell terminal differentiation versus memory formation is the Blimp-1/Bcl-6 axis [14]. We saw a reduced level of Blimp-1 mRNA and an increased level of Bcl-6 mRNA in $CD8^+$ T cells lacking STAT5 compared to wildtype (Table 4.1). Interestingly, STAT5 has been shown to inhibit T_{FH} differentiation in effector $CD4^+$ T cells through increasing Blimp-1 expression and ultimately negatively regulating Bcl-6, which drives T_{FH} fate [47, 48]. When we examined protein levels of Bcl-6, we did not see differences (Fig 4.4C), but it remains possible that STAT5 impacts Blimp-1/Bcl-6 activity and thus may favor effector CTL differentiation in a manner similar to what is seen for $CD4^+$ T cells.

To test effector function more directly, we compared target cell lysis of STAT5 WT and STAT5 CKO $CD8^+$ T cells on day 8 postinfection. We found approximately a 2-fold decrease in killing on a per cell basis in the absence of STAT5 (Fig 4.4D). This could be potentially explained by the lower levels of Perforin (*Prf1*) mRNA expressed by STAT5

CKO CTL compared to wild-type CTL (Table 4.1) and is consistent with a report that STAT5 binds the Prf1 promoter and drives Prf1 expression in CTL [4].

Over all, our data suggest that on a per cell basis, STAT5 deficiency has a modestly adverse impact on CTL effector function. This is similar to the modest decrease in primary effector function seen in CTL that do not receive IL-2 signals [3], and it is possible that IL-2 driven effector function is mediated at least to some degree by STAT5. However, taking into account that STAT5 does not affect peripheral localization of effector CTL and that STAT5 CKO CTL express GzB, cytokines, transcription factors, and FasL equal to wildtype (Fig. 4.4, Table 4.1), we conclude that STAT5 plays only a modest role in the development of primary effector CTL function.

CTL differentiation is heterogeneous, including effector and memory precursor $CD8^+$ T cell subsets that form part of the overall effector CTL pool. These two populations can be distinguished based on cell surface expression of KLRG1 and IL7R α [9]. We and others have shown that IL-2 signals drive differentiation of terminal effector phenotype CTL during the primary response [2, 3]. To determine if IL-2 driven effector differentiation could be mediated by STAT5, we examined the differentiation of STAT5 CKO antigen specific $CD8^+$ T cells based on IL7R α and KLRG1 expression following LCMV infection. We found that total numbers of STAT5 CKO $IL7R\alpha^{lo}$ $KLRG1^{hi}$ phenotype $CD8^+$ T cells were lower than STAT5 WT on day 8 postinfection, after contraction, and into memory (Fig. 4.5A). However, whereas $CD8^+$ T cells that do not receive IL-2 signals have reduced terminal effector phenotype CTLs, yet normal numbers of memory precursor and memory $CD8^+$ T cells [3], we also found reduced numbers of $IL7R\alpha^{hi}KLRG1^{lo}$ phenotype $CD8^+$ T cells in the absence of STAT5 (Fig. 4.5B). Additionally, while a proportion of $IL7R\alpha^{lo}KLRG1^{hi}$ STAT5 WT still remain on day 40 postinfection, this population within STAT5 CKO is nearly undetectable at this time

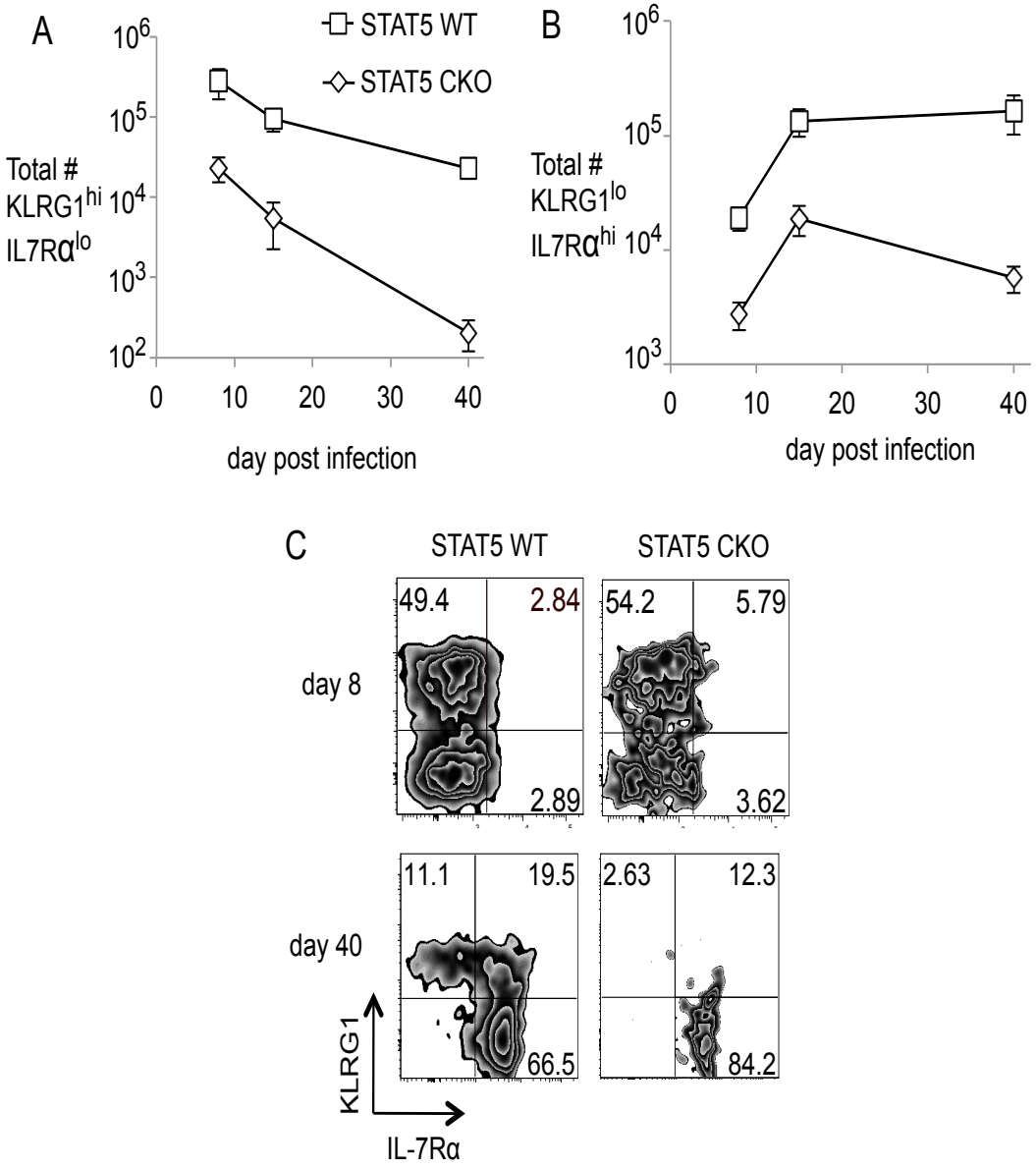

Figure 4.5 STAT5 signals during primary expansion are broadly important for all CD8+ T cells responding to acute infection. Graphs display the total number of KLRG1-hi, IL7Rα-lo (terminal effector phenotype) *(A)* and KLRG1-lo, IL7Rα-hi (memory precursor/memory phenotype) *(B)* at the indicated time points postinfection with LCMV-Arm for STAT5 WT and STAT5 CKO tetramer binding CD8+ T cells. *C,* Representative flow plots display the frequency of CD8+ T cell subsets as determined by KLRG1 and IL7Rα expression at the indicated time points for STAT5 WT and STAT5 CKO tetramer binding CD8+ T cells. Results are representative of 3-4 separate experiments. Error bars represent the SEM (n=3-4 per group).

point (Fig. 4.5C). We conclude that in contrast to IL-2, STAT5 does not selectively drive differentiation of terminal effector phenotype CTL and is instead important the emergence of all effector CTL through the peak of expansion. After clearance of pathogen, STAT5 signals are important for maintaining IL7Rα^{lo} KLRG1^{hi} phenotype CTL through contraction and into the memory phase.

STAT5 signals are important for maintenance of tissue-residing memory CTL after pathogen clearance

Memory CD8$^+$ T cell populations were detectable in the spleen in the absence of STAT5 following LCMV infection, although at lower numbers than wildtype (Fig. 4.6A). Importantly, surviving memory cells did not express detectable STAT5 (Fig. 4.7A), indicating that memory CTL can emerge in the absence of STAT5, albeit at lower levels than wildtype. We did not examine memory CD8$^+$ T cell survival and homeostasis beyond 6 weeks postinfection due to the known role of IL-15 and IL-7, in long-term memory T cell maintenance [28]. At early memory time points, the absence of STAT5 resulted in rapid loss of CD62L^{lo} effector memory CTL in the spleen (Fig. 4.6B) and the preferential and specific decline of tissue-homing effector memory CTL in the liver (Fig. 4.6C). These findings are consistent with the role of IL-2 in effector memory CTL differentiation and suggest that IL-2-driven STAT5 activation is a key driver of effector memory CTL establishment [3, 5]. After pathogen clearance, STAT5 activity is particularly important for effector memory and tissue residing memory CD8$^+$ T cell survival (Fig. 4.6B, C).

STAT5 CKO memory CD8$^+$ T cells have robust recall capacity

Because IL-2 signals during primary CTL activation are required for the formation of memory CTL capable of robust secondary responses [3, 5], we assessed the

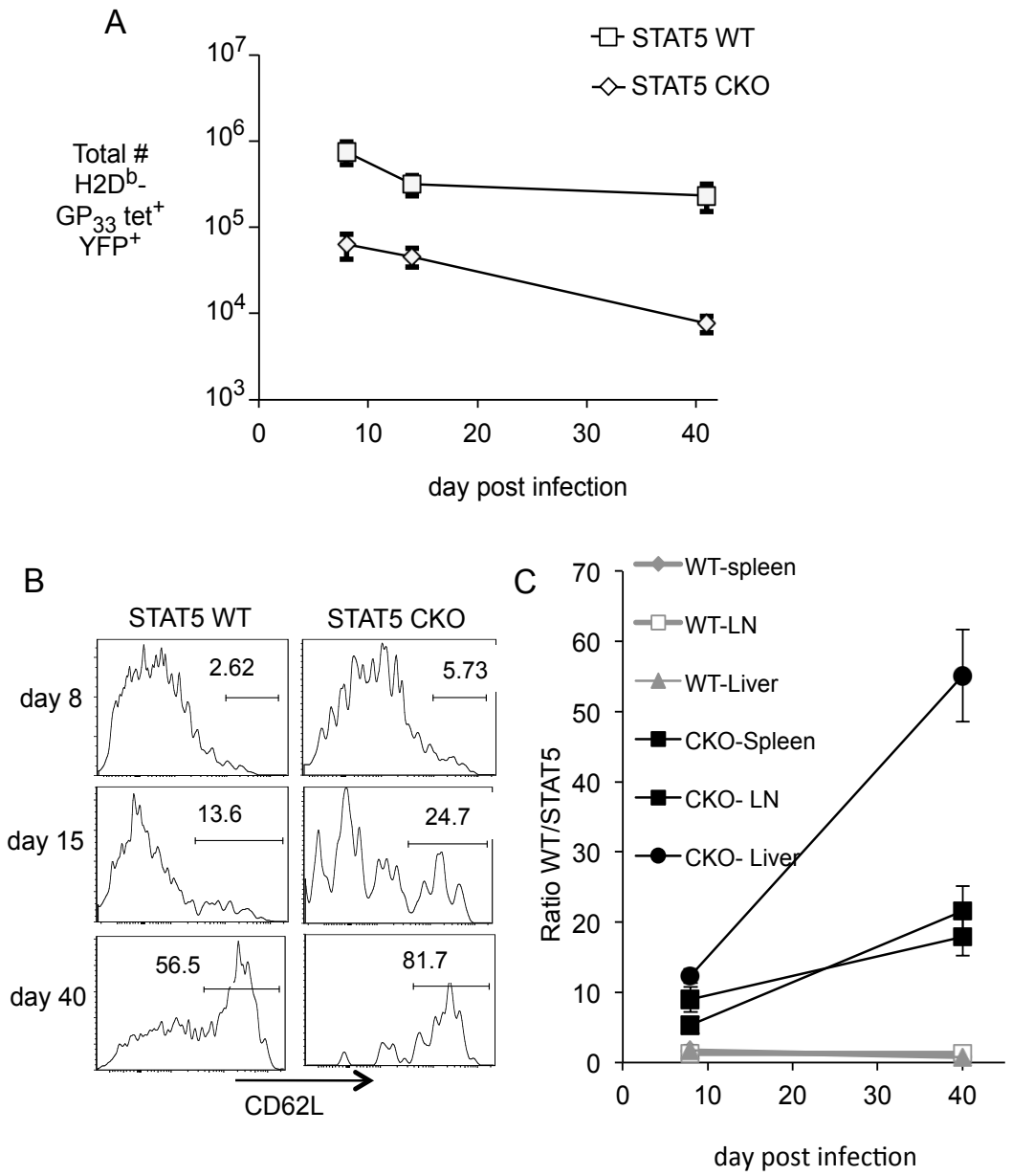

Figure 4.6 STAT5 signals are important for establishment and maintenance of effector memory and tissue residing CD8+ T cells. *A,* Total numbers of antigen specific STAT5 WT and STAT5 CKO CD8+ T cells in the spleen at the indicated time points post LCMV infection. *B,* Histograms show representative CD62L staining in STAT5 WT and CKO CD8+ T cells in the spleen at indicated time points. *C,* Ratio of H2Db-GP33-41 tetramer binding CD8+ T cells derived from WT counterpart compared to STAT5 counterpart YFP+CD8+ T cells in the indicated tissues at day 8 and day 40 postinfection. Results are representative of 3-4 independent experiments and error bars represent the SEM (n=3-4 per group).

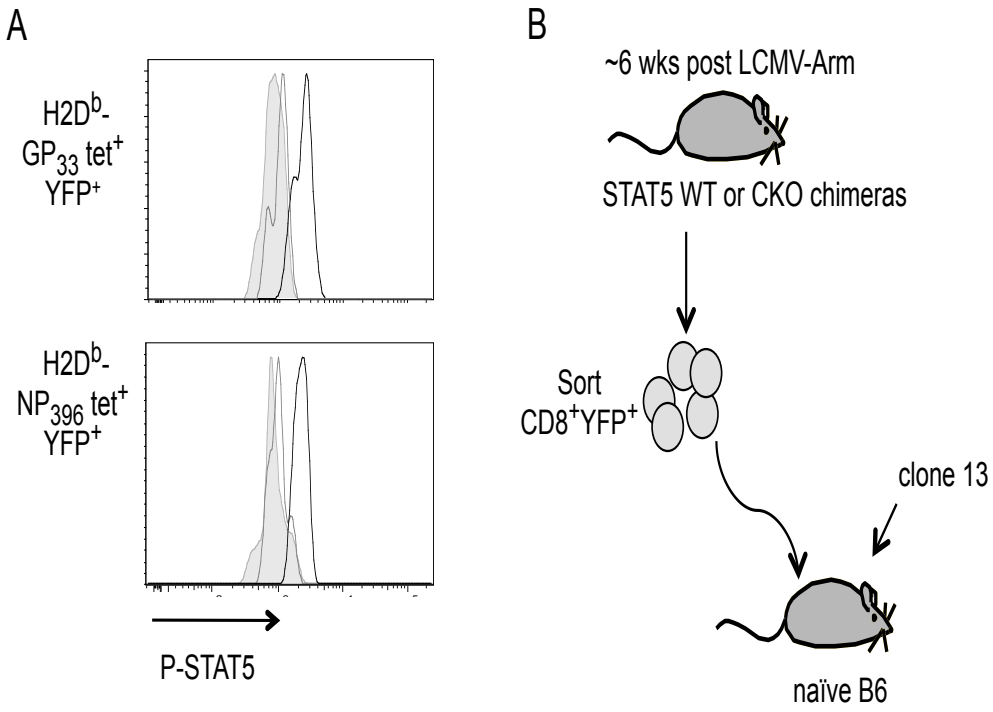

Figure 4.7 Adoptive transfer strategy to determine recall capacity of STAT5 CKO memory CD8+ T cells. *A,* Confirmation of deletion of STAT5 in memory CD8+ T cell populations. *B,* Schematic showing adoptive transfer strategy for determining recall capacity of STAT5 CKO CD8+ T cells compared to WT. CD8+YFP+ memory T cells were FACS sorted from pooled spleens derived from either STAT5 WT or STAT5 CKO chimeras approximately 6 weeks postinfection with LCMV. Sorted CD8+YFP+ T cells were analyzed for the composition of H2Db-GP33 and NP396 tetramer binding memory CD8+ T cells, then adoptively transferred into naïve B6 hosts. The next day, the naïve B6 hosts were infected with LCMV clone 13.

ability of STAT5 WT and STAT5 CKO memory CTL to respond to rechallenge. Prior to rechallenge, we verified that antigen specific memory $CD8^+YFP^+$ T cells from STAT5 CKO chimeras did not express detectable STAT5 (Fig. 4.7A). Direct rechallenge of memory phase bone marrow chimeras is difficult to interpret due to the inability to distinguish between recall responses by YFP^+ memory $CD8^+$ T cells and recruitment of YFP^- memory or naïve $CD8^+$ T cells that subsequently express YFP. Therefore, we used an adoptive transfer strategy to determine recall capacity in the absence of STAT5. STAT5 WT or STAT5 CKO $CD8^+YFP^+$ memory CTL were FACS-purified from the spleens of mixed chimeras 6 weeks after LCMV infection, analyzed for frequency of $tetramer^+$ cells and transferred into naïve B6 hosts. Twenty-four hours after adoptive transfer, the naïve B6 hosts were infected with LCMV clone 13 to induce recall responses by YFP^+ memory $CD8^+$ T cells (Fig 4.7B). Similar experiments were done using LCMV Armstrong or recombinant *Listeria monocytogenes* expressing GP_{33-41} (Lm-gp33) as a secondary stimulus.

Antigen-specific STAT5 WT and STAT5 CKO YFP^+CD8^+ T cells were clearly visible in the spleens of the clone 13 infected hosts at day 5 postrechallenge (Fig. 4.8A). In contrast to the primary response, STAT5 CKO memory $CD8^+$ T cells expanded at levels similar to wild-type when rechallenged (Fig. 4.8B-D). This is in striking contrast to the defective accumulation upon rechallenge of memory $CD8^+$ T cells generated in the absence of IL-2 [3, 5]. As in the primary response, STAT5 CKO secondary CTLs express normal levels of GranzymeB (Fig. 4.9A), IFN-γ (Fig. 4.9B) and differentiation-associated transcription factors T-bet and Eomes (Fig. 4.9A). When rechallenged with Lm-gp33, STAT5 CKO secondary CTLs trafficked to the liver normally (Fig. 4.9C). There is a modest decrease in formation of secondary $KLRG1^{hi}IL7R\alpha^{lo}$ phenotype $CD8^+$ T cells in the absence of STAT5 (Fig 4.9D); however, it is not clear what these markers may

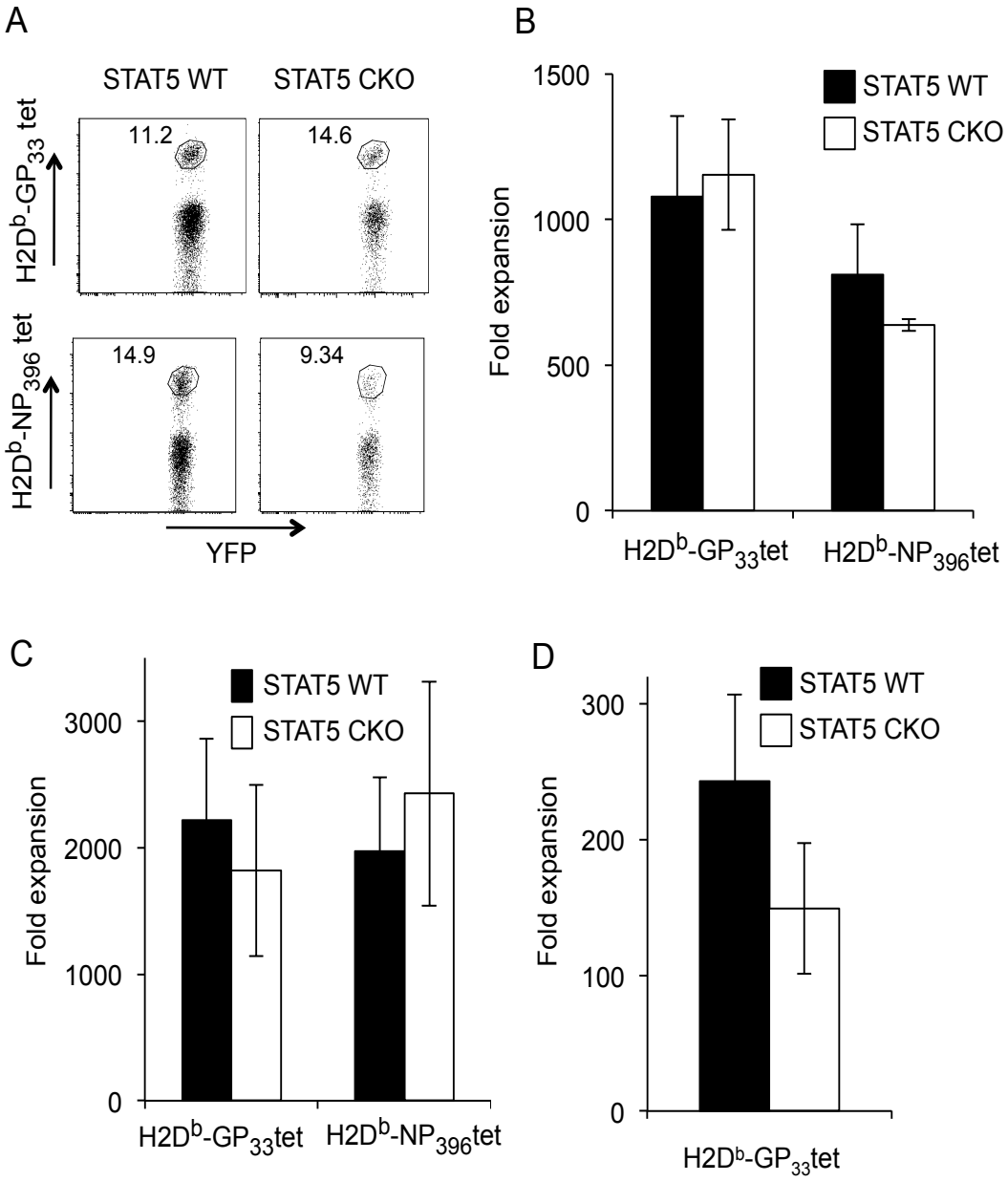

Figure 4.8 STAT5-deficient memory CD8+ T cells are capable of robust recall responses. *A,* Representative flow plots show the frequencies of antigen specific CD8+ STAT5 WT and STAT5 CKO secondary responders in the spleens of B6 hosts on day 5 postinfection with LCMV clone 13. Bar graphs display the fold expansion of antigen specific STAT5 WT and STAT5 CKO CD8+YFP+ cells on day 5 post rechallenge with *(B)* LCMV clone 13, *(C)* LCMV Armstrong, or *(D)* Lm-gp33 compared to numbers adoptively transferred prior to infection. Results are representative of 3-4 independent experiments and error bars represent the SEM (n=3-4 per group).

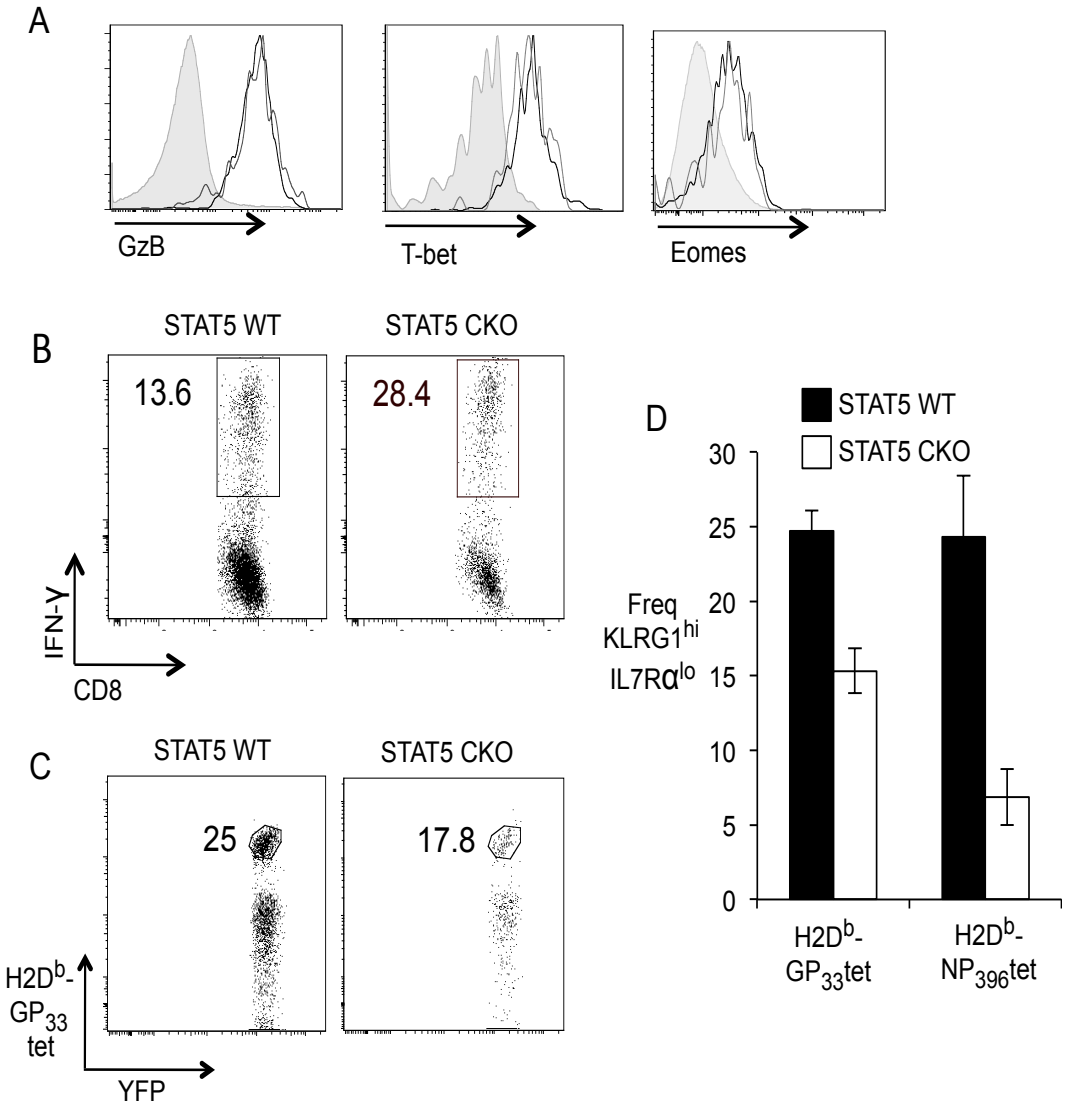

Figure 4.9 STAT5 is not required for secondary effector CTL differentiation. A, Intracellular staining of STAT5 WT (black line) and STAT5 CKO (gray line) CD8+ T cells for GranzymeB, T-bet, and Eomes on day 5 post rechallenge with LCMV clone 13 (experiment performed as described in Fig 6A). Gray shaded histograms are isotype controls. *B,* Intracellular cytokine staining for IFN-γ was performed after ex vivo restimulation on day 5 postrechallenge. Splenocytes were restimulated with GP33-41 or NP396-404 peptides in the presence of Golgi Plug. *C,* Representative flow plots show the presence of secondary CTL in the liver of Lm-gp33 infected mice on day 5 postinfection. *D,* Bar graphs show the frequency of secondary KLRG1-hi, IL7Rα-lo phenotype CD8+ T cells within YFP+ populations on day 5 postrechallenge. Results are representative of 3-4 separate experiments. Error bars are SEM (n=3-4 per group).

indicate of in terms of secondary CD8$^+$ T cell memory differentiation. Thus, secondary effector CTL differentation does not require STAT5. Overall, we conclude that IL-2-driven programming of memory CD8$^+$ T cells capable of robust secondary expansion is largely STAT5 independent.

Discussion

Our results demonstrate a broad role for STAT5 signaling in the primary response to infection in promoting the accumulation of effector CTL, and a more selective role for STAT5 in the survival of effector phenotype and establishment and survival of tissue residing memory CD8$^+$ T cells after pathogen clearance. A study using direct infection of Mx1-Cre Stat5$^{fl/fl}$ mice showed that BRDU incorporation of wildtype and STAT5-deficient CD8$^+$ T cells on day 8 postinfection was the same [36], which suggests that differences in overall expansion seen in our experiments are likely due to poor survival of STAT5-deficient CD8$^+$ T cells. Additionally, the absence of IL-2 signaling during the in vivo response to acute infection impacts survival while having little effect on cell division [5]. However, differences in early proliferation (prior to day 5) could also contribute to lower expansion in the absence of STAT5.

Consistent with reduced survival in the absence of STAT5, we did see reduced expression of the pro-survival molecule Bcl-$_X$L in STAT5 CKO CD8$^+$ T cells at day 8 compared to WT, which was also seen for CD8$^+$ T cells over that do not receive IL-2 signals [3]. It is known that STAT proteins, including STAT5, can induce Bcl-xL in several cell types [49]. It is possible that IL-2 induced STAT5 could promote survival of CD8$^+$ T cells during the primary response through induction of Bcl-xL. However, T cells lacking Bcl-xL (all isoforms) were able to mount normal responses to *Listeria monocytogenes* infection [50]. Additionally, Bcl-xL can be regulated post-translationally.

Thus, the impact of differences in Bcl-$_X$L mRNA levels during primary expansion in the absence of IL-2 and STAT5 signals are not clear.

We find that STAT5 signals are broadly important for all effector CTL cell during primary expansion, suggesting that STAT5 does not drive CD8$^+$ T cell fate decisions during the primary response. This is in contrast to the specific role of IL-2 in preferentially promoting the differentiation of terminal effector phenotype CTL [3]. Another γ_c family member, IL-21, also able to activate STAT5, though to a lesser degree than IL-2 [51], has recently been identified as being important in CD8$^+$ T cell differentiation in the context of chronic [52-54] and acute infection [55]. Additional STAT5 activation during the primary response is likely stimulated by IL-7 and IL-15, and we have found that the combined absence of IL-2 and IL-15 enhances the defect in terminal effector CTL emergence during the primary response [3]. Our results suggest that STAT5 may have graded effects on CTL differentiation. While the loss of potent STAT5 activation induced by IL-2 preferentially impacts terminal effector phenotype CTL formation, STAT5 activation induced by other cytokines may be sufficient for the differentiation of memory precursor CTL. Complete loss of STAT5, however, results in a defect in both terminal effector phenotype and memory precursor CTL. Therefore, while complete STAT5 deficiency does not reveal a binary role for STAT5 in promoting the differentiation of one subset or the other, our results suggest that the extent and/or duration of STAT5 activation impacts CD8$^+$ T cell subset differentiation during the primary response, as well as providing broad survival signals during expansion.

The cytokine IL-15 selectively promotes survival of terminal effector phenotype CTL after viral clearance [3, 56], while IL-7 promotes memory precursor phenotype CD8$^+$ T cell survival during contraction [56]. Although both of these cytokines induce STAT5 signaling, we observed a switch from a broad requirement for STAT5 during expansion to a more selective requirement for its activity in the maintenance of effector

phenotype and long lived effector memory CD8$^+$ T cells during contraction and into the memory phase. This could be due to different levels of STAT5 activation induced by IL-15 and IL-7 [57]. Alternatively, another report has shown that effector phenotype CD8$^+$ T cells are more dependent on STAT5 signals for survival during contraction [35, 36]. Collectively, these reports and ours emphasize that for different CD8$^+$ T cells subsets, there appears to be differential usage of and access to common signaling cascades induced by common gamma chain cytokines.

Importantly, we observed the emergence of a STAT5-deficient memory CD8$^+$ T cell population 6 weeks after infection, albeit at lower levels than what was seen for wildtype. This brings to question the survival signals that are used in the absence of STAT5 and to what degree these signals are used under normal conditions. A recent report suggests that STAT5-independent survival signals could be utilized in the formation of memory CD8$^+$ T cell populations [57], but the nature of those signals remain unknown. A recent study showed that memory precursor CTLs were able to activate IL-15-induced PI3K/Akt more efficiently than effector phenotype CTLs [35]. However, hyper-activation of this pathway was detrimental to long term survival of memory CD8$^+$ T cells.

Despite being a key mediator of IL-2 signals, we found that STAT5 was dispensable for the ability of memory CD8$^+$ T cells to mount robust secondary responses and undergo secondary effector CTL differentiation. This highlights a different requirement for STAT5 during primary and secondary CD8$^+$ T cell responses, as STAT5-deficient primary responses were reduced compared to wildtype. Moreover, this is in striking contrast as to what is seen for memory CD8$^+$ T cells generated in the absence of IL-2 signals, which fail to mount successful recall responses and fail to differentiate into secondary effector CTL [3, 5]. Thus, the mechanism by which IL-2 signals during the

primary response program memory $CD8^+$ T cells is independent of STAT5 and remains to be defined.

Alternative candidate pathways induced by IL-2 that could induce effective memory CTL differentiation include STAT3 and PI3K/Akt. STAT3 signals are also induced by IL-2 [51], and a role for STAT3 in persistence of memory precursor CTL in mice [55] and $CD8^+$ T_{CM} formation in humans [58] has been suggested. However, STAT3 is efficiently activated by a variety of other cytokines [51, 59], and it seems unlikely that a major proportion of STAT3 activity during the primary CTL response is IL-2-dependent. Moreover, the phenotypes of STAT3 deficient and IL-2Rα deficient $CD8^+$ T cells do not align, as IL-2 is important for terminal effector CTL [3] whereas STAT3 appears to be important for memory precursor phenotype $CD8^+$ T cells [55].

A more likely candidate is IL-2-dependent PI3K/Akt activation. While this signaling pathway is induced in $CD8^+$ T cells by a variety of sources, the additive effects of multiple signals, including IL-2, in the induction of PI3K/Akt activation for memory $CD8^+$ T cell differentiation could be important. Moreover, several recent reports have suggested that the kinase mTOR, which is downstram of PI3K/Akt activation, is a key regulator of effector and memory CTL fate decisions and survival [60-62]. Therefore, it seems likely that IL-2 mediates its effect of CTL differentiation at least in part through PI3K/Akt activation.

References

1. Cox, M.A., L.E. Harrington, and A.J. Zajac, *Cytokines and the inception of CD8 T cell responses*. Trends in immunology, 2011. **32**(4): p. 180-6.

2. Kalia, V., et al., *Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo*. Immunity, 2010. **32**(1): p. 91-103.

3. Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells*. Journal of immunology, 2010. **184**(12): p. 6719-30.

4. Pipkin, M.E., et al., *Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells*. Immunity, 2010. **32**(1): p. 79-90.

5. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells*. Nature, 2006. **441**(7095): p. 890-3.

6. Cruz-Guilloty, F., et al., *Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs*. The Journal of experimental medicine, 2009. **206**(1): p. 51-9.

7. Intlekofer, A.M., et al., *Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells*. The Journal of experimental medicine, 2007. **204**(9): p. 2015-21.

8. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin*. Nature immunology, 2005. **6**(12): p. 1236-44.

9. Joshi, N.S., et al., *Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor*. Immunity, 2007. **27**(2): p. 281-95.

10. Sullivan, B.M., et al., *Antigen-driven effector CD8 T cell function regulated by T-bet*. Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(26): p. 15818-23.

11. Yeo, C.J. and D.T. Fearon, *T-bet-mediated differentiation of the activated CD8+ T cell*. European journal of immunology, 2011. **41**(1): p. 60-6.

12. Banerjee, A., et al., *Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche*. Journal of immunology, 2010. **185**(9): p. 4988-92.

13. Pearce, E.L., et al., *Control of effector CD8+ T cell function by the transcription factor Eomesodermin*. Science, 2003. **302**(5647): p. 1041-3.

14. Crotty, S., R.J. Johnston, and S.P. Schoenberger, *Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation*. Nature immunology, 2010. **11**(2): p. 114-20.

15. Ji, Y., et al., *Repression of the DNA-binding inhibitor Id3 by Blimp-1 limits the formation of memory CD8+ T cells*. Nature immunology, 2011. **12**(12): p. 1230-7.

16. Kallies, A., et al., *Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses*. Immunity, 2009. **31**(2): p. 283-95.

17. Rutishauser, R.L., et al., *Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties*. Immunity, 2009. **31**(2): p. 296-308.

18. Shin, H., et al., *A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection*. Immunity, 2009. **31**(2): p. 309-20.

19. Ichii, H., et al., *Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells*. Nature immunology, 2002. **3**(6): p. 558-63.

20. Ichii, H., et al., *Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells*. Journal of immunology, 2004. **173**(2): p. 883-91.

21. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*)*. Annual review of immunology, 2010. **28**: p. 445-89.

22. Malek, T.R., *The biology of interleukin-2*. Annual review of immunology, 2008. **26**: p. 453-79.

23. Kane, L.P. and A. Weiss, *The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3*. Immunological reviews, 2003. **192**: p. 7-20.

24. Okkenhaug, K., et al., *Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice*. Science, 2002. **297**(5583): p. 1031-4.

25. Song, J., et al., *Intracellular signals of T cell costimulation*. Cellular & molecular immunology, 2008. **5**(4): p. 239-47.

26. Ashwell, J.D., *The many paths to p38 mitogen-activated protein kinase activation in the immune system*. Nature reviews. Immunology, 2006. **6**(7): p. 532-40.

27. Schluns, K.S. and L. Lefrancois, *Cytokine control of memory T-cell development and survival*. Nature reviews. Immunology, 2003. **3**(4): p. 269-79.

28. Surh, C.D. and J. Sprent, *Homeostasis of naive and memory T cells*. Immunity, 2008. **29**(6): p. 848-62.

29. Haring, J.S., et al., *Constitutive expression of IL-7 receptor alpha does not support increased expansion or prevent contraction of antigen-specific CD4 or CD8 T cells following Listeria monocytogenes infection*. Journal of immunology, 2008. **180**(5): p. 2855-62.

30. Hand, T.W., M. Morre, and S.M. Kaech, *Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection*. Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(28): p. 11730-5.

31. Imada, K., et al., *Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity*. The Journal of experimental medicine, 1998. **188**(11): p. 2067-74.

32. Nakajima, H., et al., *An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction.* Immunity, 1997. **7**(5): p. 691-701.

33. Yao, Z., et al., *Stat5a/b are essential for normal lymphoid development and differentiation.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(4): p. 1000-5.

34. Seki, Y., et al., *IL-7/STAT5 cytokine signaling pathway is essential but insufficient for maintenance of naive CD4 T cell survival in peripheral lymphoid organs.* Journal of immunology, 2007. **178**(1): p. 262-70.

35. Hand, T.W., et al., *Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(38): p. 16601-6.

36. Tripathi, P., et al., *STAT5 is critical to maintain effector CD8+ T cell responses.* Journal of immunology, 2010. **185**(4): p. 2116-24.

37. Jacob, J. and D. Baltimore, *Modelling T-cell memory by genetic marking of memory T cells in vivo.* Nature, 1999. **399**(6736): p. 593-7.

38. Srinivas, S., et al., *Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus.* BMC Dev Biol, 2001. **1**: p. 4.

39. Cui, Y., et al., *Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation.* Molecular and cellular biology, 2004. **24**(18): p. 8037-47.

40. Ahmed, R., et al., *Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence.* The Journal of experimental medicine, 1984. **160**(2): p. 521-40.

41. Shen, H., et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(9): p. 3987-91.

42. Slifka, M.K., et al., *Antiviral cytotoxic T-cell memory by vaccination with recombinant Listeria monocytogenes.* J Virol, 1996. **70**(5): p. 2902-10.

43. Williams, M.A., E.V. Ravkov, and M.J. Bevan, *Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory.* Immunity, 2008. **28**(4): p. 533-45.

44. Williams, M.A. and M.J. Bevan, *Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells.* Journal of immunology, 2004. **173**(11): p. 6694-702.

45. Altman, J.D., et al., *Phenotypic analysis of antigen-specific T lymphocytes.* Science, 1996. **274**(5284): p. 94-6.

46. Murali-Krishna, K., et al., *Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection*. Immunity, 1998. **8**(2): p. 177-87.

47. Johnston, R.J., et al., *STAT5 is a potent negative regulator of TFH cell differentiation*. The Journal of experimental medicine, 2012. **209**(2): p. 243-50.

48. Nurieva, R.I., et al., *STAT5 Protein Negatively Regulates T Follicular Helper (Tfh) Cell Generation and Function*. The Journal of biological chemistry, 2012. **287**(14): p. 11234-9.

49. Grad, J.M., X.R. Zeng, and L.H. Boise, *Regulation of Bcl-xL: a little bit of this and a little bit of STAT*. Current opinion in oncology, 2000. **12**(6): p. 543-9.

50. Zhang, N. and Y.W. He, *The antiapoptotic protein Bcl-xL is dispensable for the development of effector and memory T lymphocytes*. Journal of immunology, 2005. **174**(11): p. 6967-73.

51. Kovanen, P.E. and W.J. Leonard, *Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways*. Immunological reviews, 2004. **202**: p. 67-83.

52. Elsaesser, H., K. Sauer, and D.G. Brooks, *IL-21 is required to control chronic viral infection*. Science, 2009. **324**(5934): p. 1569-72.

53. Frohlich, A., et al., *IL-21R on T cells is critical for sustained functionality and control of chronic viral infection*. Science, 2009. **324**(5934): p. 1576-80.

54. Yi, J.S., M. Du, and A.J. Zajac, *A vital role for interleukin-21 in the control of a chronic viral infection*. Science, 2009. **324**(5934): p. 1572-6.

55. Cui, W., et al., *An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells*. Immunity, 2011. **35**(5): p. 792-805.

56. Rubinstein, M.P., et al., *IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response*. Blood, 2008. **112**(9): p. 3704-12.

57. Kemp, R.A., et al., *Evidence of STAT5-dependent and -independent routes to CD8 memory formation and a preferential role for IL-7 over IL-15 in STAT5 activation*. Immunology and cell biology, 2010. **88**(2): p. 213-9.

58. Siegel, A.M., et al., *A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory*. Immunity, 2011. **35**(5): p. 806-18.

59. O'Sullivan, L.A., et al., *Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease*. Mol Immunol, 2007. **44**(10): p. 2497-506.

60. Araki, K., et al., *mTOR regulates memory CD8 T-cell differentiation*. Nature, 2009. **460**(7251): p. 108-12.

61. Pearce, E.L., et al., *Enhancing CD8 T-cell memory by modulating fatty acid metabolism*. Nature, 2009. **460**(7251): p. 103-7.

62. Rao, R.R., et al., *The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin*. Immunity, 2010. **32**(1): p. 67-78.

CHAPTER 5

DISCUSSION

The work in this dissertation explores the role of IL-2, the related cytokine IL-15, and the transcription factor STAT5 in $CD8^+$ T cell effector and memory fate decisions. As a result, we have a more detailed understanding of the role of cytokines and their signaling pathways in promoting effector CTL responses, the subsequent survival of $CD8^+$ T cell subsets into the memory phase, and the programming of memory $CD8^+$ T cells capable of protective recall responses.

In the case of IL-2, we find a role in both effector CTL differentiation as well as memory $CD8^+$ T cell programming. Two recent reports have also demonstrated a role for IL-2 in driving effector CTL differentiation. These studies found that effector CTL differentiation was influenced by the concentration of IL-2 following activation[1] or by the length of time activated CTLs were able to incorporate high affinity IL-2 signals [2]. Together with our report, these studies demonstrate a non-redundant role for IL-2 in enhancing effector CTL differentiation, survival and function. Our report further demonstrates that in the complete absence of high affinity IL-2 signals, secondary effector CTL differentiation is dramatically impaired.

Because our studies found a role for IL-2 in promoting secondary CTL responses in both LCMV and *Listeria* infectious model systems [3, 4], we concluded that IL-2 played a broad role in memory CTL differentiation in disparate model systems. While the role of IL-2 in promoting secondary responses following LCMV infection have also been observed by another group [5], one recent study, while also reporting a role for IL-2 in driving primary effector CTL differentiation following *Listeria* infection, found that the recall capacity of the resulting memory cells was IL-2 independent [6]. It remains possible therefore that the role of IL-2 may depend on the pathogenic stimulus. Clearly the impact of IL-2 in driving the differentiation of effector and memory CTL requires further study.

One complicating factor may be the presence of redundancy within the immune system. We examined the potential overlapping role of the cytokine IL-15 in $CD8^+$ T cell differentiation, which is highly related to IL-2 in that its receptor shares both the β and γ_c subunits of the IL-2 receptor, which are involved in signal transduction. We find that IL-15 does not drive the differentiation of effector CTL and does not have a memory $CD8^+$ T cell programming role as we have shown for IL-2. Instead, IL-15 supports the survival of terminal effector CTL after pathogen clearance. It is possible that the levels of IL-15 available to T cells differ depending on the nature of the pathogenic stimulus, and our results do not rule out a potential role for IL-15 during primary T cell differentiation if present at high enough concentrations in other infectious model systems. IL-2, on the other hand, is secreted at high levels upon activation and is probably available to differentiating CTLs throughout the primary expansion phase.

Several other cytokines, such as IL-7 and IL-21, also utilize the common gamma chain (γ_c) as a signaling component of their receptors, and these cytokines send a common TCR-independent proliferative signal via the STAT5 and STAT3 transcription factors [7]. It is not clear, however, whether the programming of functional memory cells by IL-2 represents a unique signal from IL-2 that IL-7, IL-21 or other γ_c family member cytokines are unable to deliver, or if it represents a common signal, that any member of the family could redundantly deliver during T cell activation and differentiation, were it present in sufficient amounts and its receptor expressed. One intriguing candidate member of this family with similar induction kinetics is IL-21, which, like IL-2, is largely expressed by $CD4^+$ T cells, is induced upon activation and promotes enhanced cytotoxicity by $CD8^+$ T cells [8]. Furthermore, IL-21 has been shown to induce both Blimp-1 and Bcl-6 expression during B cell activation and differentiation, suggesting the intriguing possibility that IL-21 may play an effector/memory differentiation role for T cells [9]. Recent studies found that like IL-2 [5], IL-21 signals to

T cells were not required for primary CTL expansion but did promote their ability to control chronic infection [10-12]. Moreover, a recent study suggests that STAT3, which is induced by IL-21 and to a lesser degree by IL-2, may have a programming role in $CD8^+$ T cell memory differentiation [13].

How does IL-2 fit into current models of memory $CD8^+$ T cell differentiation? The most likely model, as discussed in the Introduction, is a scenario in which activated $CD8^+$ T cells commit to a fate of death after pathogen clearance or long-lived memory early during an immune response. Within this fate decision, progressive differentiation will continue as environmental signals promote a more or less differentiated effector $CD8^+$ state and ultimately affect the potential of a particular $CD8^+$ T cell to populate the memory pool and to provide protection from reinfection. Considering that IL-2 plays a role in both effector CTL differentiation and memory $CD8^+$ T cell programming [1-4], a goldilocks principle could be imagined. An effector CTL may need to be pushed far enough into an effector differentiation program that it can "remember" or reaccess this program later in order to mount a successful recall response. Exactly how this memory of an effector program could be obtained is not known, but epigenetic changes are likely a mechanism. If too much or too strong of an IL-2 signal is received by a particular CTL, this may drive complete terminal differentiation resulting in death after pathogen clearance or more rapid disappearance from the memory pool. Thus memory cells generated in the absence of IL-2 signals would be largely unable to enter an effector differentiation pathway upon reactivation. Future studies are needed to identify epigenetic changes as well as changes in transcriptional activity that are influenced by IL-2 signals in differentiating CTL in vivo.

IL-2 potently activates the transcription factor STAT5, and in an attempt to understand the molecular nature of the IL-2 signal delivered to $CD8^+$ T cells, we examined the role of STAT5 in $CD8^+$ T cell effector and memory differentiation. In

contrast to IL-2, which selectively drives the differentiation of terminal effector phenotype CTL, we found that STAT5 was broadly important for all $CD8^+$ T cell subsets during the primary response. In the context of our findings for both IL-2 and IL-15, our results could imply that STAT5 may have graded effects on CTL differentiation. While the loss of potent STAT5 activation induced by IL-2 preferentially impacts effector phenotype CTL formation, STAT5 activation induced by other cytokines may be sufficient for the differentiation of memory precursor CTL. Complete loss of STAT5, however, results in a defect in both effector phenotype and memory precursor CTL. Therefore, while complete STAT5 deficiency does not reveal a binary role for STAT5 in promoting the differentiation of one subset or the other, our results suggest that the extent and/or duration of STAT5 activation impacts $CD8^+$ T cell subset differentiation during the primary response, as well as providing broad survival signals during expansion.

Most strikingly, STAT5 was not required for robust recall responses and secondary effector CTL generation, which contrasts not only its own role during the primary response, but also differs from the role for IL-2 in memory $CD8^+$ T cell programming [3, 4]. Thus, we conclude that IL-2 driven memory $CD8^+$ T cell programming is independent of STAT5. The molecular nature of the IL-2 signal still remains to be defined. Alternative candidate pathways induced by IL-2 that could induce effective memory $CD8^+$ T cell differentiation include STAT3 and PI3K/Akt. STAT3 signals are also induced by IL-2 [14], and a role for STAT3 in persistence of memory precursor CTL in mice [13] and $CD8^+$ T_{CM} formation in humans [15] has been suggested. However, STAT3 is efficiently activated by a variety of other cytokines [7, 16], and it seems unlikely that a major proportion of STAT3 activity during the primary CTL response is IL-2-dependent. A more likely candidate is IL-2-dependent PI3K/Akt activation. While this signaling pathway is induced in $CD8^+$ T cells by a variety of

sources, the additive effects of multiple signals, including IL-2, in the induction of PI3K/Akt activation for memory $CD8^+$ T cell differentiation could be important. Moreover, several recent reports have suggested that the kinase mTOR, which is downstram of PI3K/Akt activation, is a key regulator of effector and memory CTL fate decisions and survival [17-19]. Therefore, it seems likely that IL-2 could mediate its effect of CTL differentiation and memory $CD8^+$ T cell programming at least in part through PI3K/Akt activation.

Our molecular understanding of $CD8^+$ T cell effector and memory differentiation remains to be enhanced. While signals to effector CTL during the primary response important for fate decisions have been identified, how these signals are incorporated and ultimately drive differentiation remains to be further explored. Further studies examining signaling pathways, transcriptional networks, and epigenetic changes that drive $CD8^+$ T cell fate decisions, as a result of IL-2 as well as other crucial signals, are essential in furthering our understanding of memory T cell biology and ultimately aid in design of vaccines and strategies aimed at immune modulation.

References

1. Pipkin, M.E., et al., *Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells.* Immunity, 2010. **32**(1): p. 79-90.

2. Kalia, V., et al., *Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo.* Immunity, 2010. **32**(1): p. 91-103.

3. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells.* Nature, 2006. **441**(7095): p. 890-3.

4. Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells.* J Immunol, 2010. **184**(12): p. 6719-30.

5. Bachmann, M.F., et al., *Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections.* Eur J Immunol, 2007. **37**(6): p. 1502-12.

6. Obar, J.J., et al., *CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses.* Proc Natl Acad Sci U S A, 2009.

7. Kovanen, P.E. and W.J. Leonard, *Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways.* Immunol Rev, 2004. **202**: p. 67-83.

8. Spolski, R. and W.J. Leonard, *Interleukin-21: basic biology and implications for cancer and autoimmunity.* Annu Rev Immunol, 2008. **26**: p. 57-79.

9. Ozaki, K., et al., *Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6.* J Immunol, 2004. **173**(9): p. 5361-71.

10. Elsaesser, H., K. Sauer, and D.G. Brooks, *IL-21 is required to control chronic viral infection.* Science, 2009. **324**(5934): p. 1569-72.

11. Frohlich, A., et al., *IL-21R on T cells is critical for sustained functionality and control of chronic viral infection.* Science, 2009. **324**(5934): p. 1576-80.

12. Yi, J.S., M. Du, and A.J. Zajac, *A vital role for interleukin-21 in the control of a chronic viral infection.* Science, 2009. **324**(5934): p. 1572-6.

13. Cui, W., et al., *An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells.* Immunity, 2011. **35**(5): p. 792-805.

14. Kovanen, P.E. and W.J. Leonard, *Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways.* Immunological reviews, 2004. **202**: p. 67-83.

15. Siegel, A.M., et al., *A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory.* Immunity, 2011. **35**(5): p. 806-18.

16. O'Sullivan, L.A., et al., *Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease.* Mol Immunol, 2007. **44**(10): p. 2497-506.

17. Araki, K., et al., *mTOR regulates memory CD8 T-cell differentiation.* Nature, 2009. **460**(7251): p. 108-12.

18. Pearce, E.L., et al., *Enhancing CD8 T-cell memory by modulating fatty acid metabolism.* Nature, 2009. **460**(7251): p. 103-7.

19. Rao, R.R., et al., *The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin.* Immunity, 2010. **32**(1): p. 67-78.